AD_____

Award Number: W81XWH-09-1-0221

TITLE: Chaperone Function in Androgen-Independent Prostate Cancer

PRINCIPAL INVESTIGATOR: BRYCE M. PASCHAL, PhD

CONTRACTING ORGANIZATION:

UNIVERSITY OF VIRGINIA
CHARLOTTESVILLE, VA 22908

REPORT DATE: May 2010

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-05-2010	Annual Report	1 May 2009 - 30 Apr 2010

4. TITLE AND SUBTITLE
Chaperone Function in Androgen-Independent Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0221

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
BRYCE M. PASCHAL, PhD

paschal@virginia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF VIRGINIA
CHARLOTTESVILLE, VA 22908

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

Approved for public release; distribution unlimited

14. ABSTRACT

We found that the Hsp90 co-chaperone, FKBP51, is upregulated at both the protein and mRNA levels in LAPC-4 A compared with LAPC-4 AD. Using biochemical approaches we found FKBP51 promotes assembly of a "superchaperone" complex containing ATP-bound Hsp90, p23, and FKBP51. Overexpression of FKBP51 leads to formation of maximal number of Apo-AR complexes, thereby increasing the number of AR molecules that bind hormone. FKBP51 enhances AR-dependent transcription of genes including PSA in response to both androgens and androgen-antagonists. Over-expression of FKBP51 enhances both AI and AD prostate cell growth in culture. Our working model is that FKBP51 determines androgen responsiveness in prostate cancer cells. By increasing the number of functional AR molecules that form in a low-androgen environment, over-expression of FKBP51 contributes to disease progression through androgen signaling and prostate cell proliferation. Drugs to FKBP51 may provide a complement or alternative to existing prostate cancer therapies.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSOI**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	24	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

INTRODUCTION:

Ligand binding to nuclear receptors including the AR is regulated by molecular chaperones. The central player in this reaction is Hsp90, an ATP-utilizing chaperone that interacts transiently with LBDs to stabilize a conformation that is appropriate for ligand binding (Pratt and Toft, 1997b). Less well understood are the co-chaperones that belong to the immunophilin class of proteins, FK506-binding proteins FKBP51 and FKBP52, and cyclosporin A binding protein Cyp40. These co-chaperones have N-terminal domains with peptidyl-prolyl isomerase activity, and C-terminal domains that contain three tetratricopeptide repeats (TPR) that mediate binding to Hsp90 (Ratajczak et al., 2003). The functions of FKBP51 and FKBP52, which are 70% identical at the protein level, have been studied mostly in the context of the PR and glucocorticoid receptor (GR). FKBP51 negatively regulates GR and PR activity by reducing hormone binding affinity (Denny et al., 2000). In contrast, FKBP52 enhances GR, PR, and AR responsiveness to cognate hormone (Riggs et al., 2003). FKBP52 knockout mice have developmental defects in reproductive tissues (in males) consistent with reduced AR signaling and a failure of embryo implantation (in females) consistent with reduced PR signaling (Cheung-Flynn et al., 2005; Yong et al., 2007b). These observations together with the apparent absence of a phenotype in mice lacking FKBP51 led to the conclusion that FKBP51 does not play a significant role in AR signaling (Yong et al., 2007b). Here we show the Hsp90 co-chaperone FKBP51 is over-expressed in a xenograft model of AI prostate cancer, and describe a molecular mechanism for how FKBP51 promotes AR signaling in prostate cancer cells.

BODY:

PROGRESS WITH RESPECT TO THE STATEMENT OF WORK

Task 1 Optimize FKBP51 staining on paraffin-embedded samples (month 1).
Progress: We have generated additional antibodies to recombinant FKBP51, which are being characterized to determine if they are sufficiently robust and specific to allow FKBP51 detection in paraffin embedded material. This characterization is critical because TMAs are precious reagents.

Task 2 FKBP51 immunocytochemistry on in-house TMA (months 1-3).
Progress: This task has not been initiated because it requires completion of TASK 1.

Task 3 FKBP51 immunocytochemistry on TMA from NCI (months 1-3).
Progress: This task has not been initiated because it requires completion of TASK 2.

Task 4 Statistical analysis of TMA results (month 4).
Progress: This task has not been initiated because it requires completion of TASK 3.

Task 5 Characterize growth rates of FKBP51 over-expressing lines in culture (low androgen, anti-androgens) (months 4-10).
Progress: This task has been completed (see paragraph 3).

Task 6 Measure sub-cutaneous tumor growth using FKBP51 lines (months 6-12).
Progress: We have performed a pilot experiment using the using FKBP51 lines; a larger experiment (more mice) will be performed to gather quantitative data.

Task 7 Intra-femoral injections with FKBP51 C4-2b lines, measure bone changes by Faxitron (months 10-16).
Progress: This experiment has not been performed because we want to complete the sub-cutaneous growth experiments before the bone experiments.

Task 8 Quantitative histomorphometry on bone experiments (months 16-21).

Progress: This TASK has not been performed because it depends on completion of the bone experiments.

Task 9 Test FKBP51 shRNA lines for sub-cutaneous and bone growth (months 18-24).
Progress: The cell lines needed for this experiment have been generated and partly characterized (see paragraph 4).

Task 10 Determine androgen sensitivity of FKBP51 lines, sub-cutaneous and in bone (months 18-21).
Progress: This work has not been initiated.

Task 11 Refine and repeat experiments, write manuscript, submit for publication (months 21-24).
Progress: This TASK has been completed (see paragraph 1).
Specific Aim 2.

Task 12 Use ligand binding assays to measure estrogen binding to AR (month 25).
Progress: This work has not been initiated.

Task 13 Use competition assays to determine Bmax and Ki values for anti-androgens in response to FKBP51 (months 25-27)**.**
Progress: Pilot experiments using a protein interaction assay described in Li et al 2010 were performed; a more rigorous approach with radiolabeled ligand is now required.

Task 14 Use proteolytic digestion to measure Hsp90 conformational changes (months 26-27).
Progress: This work has not been initiated owing to some minor difficulties with recombinant protein preparation, but we should be able to overcome the technical challenges.

Task 15 Determine if FKBP51 increases Hsp90 affinity for AR by biochemical reconstitution (months 26-28).
Progress: This TASK was partially completed and published (see paragraph 6).

Task 16 Test whether FKBP51 regulates the ATPase cycle of Hsp90 by nucleotide exchange (month 27-29).
Progress: This work has not been initiated.

Task 17 Determine if FKBP51 inhibition sensitizes Hsp90 to 17-AAG for cell growth (months 29-33).
Progress: This TASK was completed (see paragraph 12).

Task 18 Determine if FKBP51 inhibition sensitizes Hsp90 to 17-AAG for androgen binding and AR-dependent transcription (months 29-33).
Progress: This work has not been initiated.

Task 19 Refine and repeat experiments, write manuscript, submit for publication (months 33-36).
Progress: This work has not been initiated.

PARAGRAPH 1. *FKBP51 is over-expressed in a xenograft model of AI prostate cancer.* We reasoned that chaperones involved in regulating the ligand binding cycle of AR might be altered during prostate cancer progression and promote AI or androgen-hypersensitive growth. We tested this hypothesis by comparing expression levels of chaperones in LAPC-4 xenografts propagated in *scid* mice (Klein et al., 1997). LAPC-4 AD and AI cells were grown in intact and castrated mice, respectively. By immunoblotting the tumor extracts, the levels of Hsp90, Hsp70, FKBP52, Cyp40, and p23 were comparable in LAPC-4 AD and AI samples (Fig. 1A). In contrast, FKBP51 protein was elevated ~3-fold in the LAPC-4 AI relative

to AD (FIG. 1A, Appendix). Immunocytochemistry with a monoclonal antibody to FKBP51 confirmed that FKBP51 overexpression occurs in the LAPC-4 AI tumors (FIG. 1B, Appendix). Real-time PCR was used to show there is a 7 to 10-fold increase in FKBP51 mRNA (FIG. 1C, Appendix). PSA transcript levels, which normally are tightly regulated by AR and androgen, were slightly higher in 2 of 3 AI xenografts (FIG. 1C, Appendix). Another androgen-regulated gene, S100P, was upregulated 6 to 15-fold in the AI xenografts (FIG. 1C, Appendix). We interpreted these data as provisional evidence for a mechanism that promotes AR activity by compensating for the reduced androgen environment of a castrated host. FKBP51 levels are also elevated in the AI xenograft generated from the AD xenograft CWR22 (Febbo et al., 2005). Thus, FKBP51 is overexpressed in the two most commonly used xenograft models of AI prostate cancer. During the funding period, we refined and repeated experiments shown as preliminary data in the proposal, wrote the manuscript, submitted it for publication, and performed additional experiments needed for acceptance in the journal *Molecular and Cellular Biology* (Li et al., 2010)(**TASK 11**). While our MCB manuscript contains data from experiments that were not explicitly featured as TASKS in the Statement of Work, the data contributes to the larger body of work that was necessary to create a publishable story, the work was done with DOD funding for Dr. Li, and many of the findings were confirmed and refined with DOD funding.

PARAGRAPH 2. *FKBP51 Positively Regulates AR activity measured with reporter genes.* FKBP51 is an androgen-regulated gene in prostate cancer cells (Magee et al., 2006). FKBP51 over-expression in the AI xenografts LAPC-4 and CWR22Rv1 could, therefore, be a result of enhanced AR signaling characteristic of the AI state. We considered the alternative hypothesis, namely, that FKBP51 over-expression enhances AR function and thereby facilitates the AI-type phenotype. To address this hypothesis we tested whether FKBP51 can modulate AR activity. We assayed AR-dependent transcription in the absence and presence of co-transfected FKBP51 in the prostate cancer cell line PC-3, which are negative for both AR and FKBP51. Re-introduction of AR into PC-3 cells activates the endogenous FKBP51 gene, an observation that is consistent with AR regulation of FKBP51 expression (Kesler et al., 2007). FKBP51 expression resulted in a strong enhancement of androgen-stimulated transcription from a reporter gene containing the 6 Kb PSA promoter, also consistent with a previous report (Febbo et al., 2005). There was no obvious effect on AR protein levels (FIG. 2A, Appendix), a good indication that the FKBP51 effect on androgen-stimulated transcription is not simply a chaperone effect on AR protein stabilization. Additional evidence that FKBP51 regulates AR activity was obtained by showing it enhances androgen-stimulated transcription from other promoters known to be regulated by AR. FKBP51 enhanced androgen-stimulated transcription measured on AREs from the Probasin (Plymate et al., 2004) promoter (AAR3tk-Luc; FIG. 2B, Appendix), the MMTV promoter (FIG. 2C, Appendix), and from synthetic AREs (FIG. 2E, Appendix). FKBP51 clearly has opposite effects on AR and GR, as FKBP51 inhibited dexamethasone (DEX)-stimulated GR transcription from the MMTV promoter (FIG. 2D, Appendix; (Denny et al., 2000)). FKBP51 enhancement of AR activity was inhibited by treating cells with FK506, a ligand that binds with sub-micromolar affinity to FKBP51 (FIG. 2F, Appendix;(Yeh et al., 1995)). FKBP52 was also capable of enhancing AR activity in our assays, though unlike FKBP52, its effect was not inhibited significantly by FK506. This result is consistent with studies from Smith and co-workers who showed that FKBP52 regulation of steroid hormone receptor activity is independent of its enzyme activity (Riggs et al., 2007).

PARAGRAPH 3. *Levels of FKBP51 are important for transcription of endogenous AR-regulated genes.* To address the contribution of FKBP51 to endogenous gene expression, we used lentivirus to generate LNCaP cell lines that stably express FLAG-FKBP51 with LacZ as a control. FLAG-FKBP51 enhanced androgen-dependent expression of the endogenous *PSA* gene measured by real-time PCR (Fig. 3A, Appendix). Since AR and androgens are critical regulators of prostate cell growth, we examined whether FKBP51 expression promotes androgen-dependent cell growth (**TASK 5**). We assayed growth of the LNCaP-FKBP51 and LNCaP-lacZ lines in the presence of androgen over six days (Fig. 3B, Appendix). In the absence of androgen, relatively growth of LNCaP-lacZ and LNCaP-FKBP51 cells was observed. Both lines underwent growth in response to a low concentration (50 pM) of the synthetic androgen

R1881, however, FKBP51 expression provided an advantage for cell growth in this assay ($p < 0.001$; FIG. 3B, Appendix, **TASK 5**). We have done an initial round of

PARAGRAPH 4. Because the FKBP51 mouse knockout does not display overt defects in androgen signaling (Cheung-Flynn et al., 2005; Yong et al., 2007a), the contribution of FKBP51 to AR function has been unclear. We considered the possibility that FKBP51 is dispensable in normal cells, but that FKBP51 might be important for AR activity in prostate cancer cells. We tested whether androgen signaling through AR is dependent on endogenous FKBP51 by a knockdown approach (**TASK 9**), using lentivirus to transduce LNCaP and CWR22Rv1 cells with shRNA targeted to FKBP51. Lentivirus containing shRNA targeted to GFP was used as a control (part of TASK 9). Real-time PCR was used to measure androgen induction of AR-regulated genes in control and knockdown cell lines. In cells containing the FKBP51 shRNA, FKBP51 transcript and protein levels after androgen treatment (24 hrs) were reduced to ~50% of control cells (FIG. 3C, E, Appendix). There was a corresponding reduction (~50%) in the transcripts levels for the *KLK2*, *PSA*, and *S100P* genes. There was no significant effect on the AR-regulated *TMPRSS2* gene (FIG. 3C, Appendix). Transcript levels for the *KLK2, PSA,* and *S100P* genes were reduced in CWR22Rv1 cells that contained the FKBP51 shRNA (FIG. 3D, Appendix, an AI derivative of CWR22 that over-expresses FKBP51 (Nagabhushan et al., 1996). These data provide clear evidence that FKBP51 contributes to the regulation of AR activity in prostate cancer cells. This is despite the fact that FKBP51 is not required for AR function in development of the male reproductive system in the mouse, which instead relies on the closely related co-chaperone FKBP52 (Cheung-Flynn et al., 2005; Yong et al., 2007a).

PARAGRAPH 5. *FKBP51 Expression Enhances AR Responsiveness to Diverse Ligands.* Over-expression of AR increases transcription and growth in response to anti-androgen Casodex (Cas)(Chen et al., 2004). Reasoning that FKBP51 regulates some aspect of the AR ligand binding cycle, we analyzed whether its over-expression alters AR-dependent transcription in response to several AR-binding ligands. FKBP51 stimulated AR transcriptional activity in the presence of Cas, cyproterone acetate (CPA), and hydroxyflutamide (OH-Flu), while little enhancement was observed in the presence of flutamide (Flu) (FIG. 4A, Appendix). FKBP51 also stimulated AR activity in the presence of the adrenal androgen androstenedione (ASD) and high concentrations of estrogen (E2), but not of dexamethasone (Dex; FIG. 4A, Appendix). These results prompted us to explore whether FKBP51 affects the efficacy of the anti-androgen Cas, which is used as a competitive inhibitor of adrenal androgens in patients undergoing androgen ablation therapy. We tested this by measuring AR transcription activity in the presence of synthetic androgen (R1881) and a range of Cas concentrations. While FKBP51 did not change the apparent K_i for Cas, it did render AR resistant to maximal inhibition by Cas (FIG. 4B, Appendix, part of TASK 13). In fact, the level of AR activity measured in the presence of FKBP51 over-expression and a saturating concentration of Cas (10-100 mM) was comparable to the level of AR activity measured simply in the presence of R1881 (gray shading). These data show that FKBP51 over-expression promotes AR activity in response to a variety of ligands, and it has the potential to influence the efficacy of anti-androgens used in prostate cancer treatment.

PARAGRAPH 6. *FKBP51 expression alters the chaperone composition of AR complexes.* To gain insight into how FKBP51 regulates AR, we analyzed the chaperone composition of AR complexes from PC-3 cells co-transfected with FKBP51. Flag-AR complexes were isolated by immunoprecipitation (IP) under non-denaturing conditions and molybdate was included to preserve chaperone interactions. Chaperones that co-precipitated with flag-AR included Hsp90, Hsp70, FKBP51, and p23 (Fig. 5A, Appendix). FKBP51 induced a striking increase in the level of chaperones in AR complexes, particularly p23 and Hsp90 (FIG. 5A, Appendix, **part of TASK 15**). We viewed this effect as potentially significant because the co-chaperone p23 binds directly to the ATP-form of Hsp90, and this is known to stabilize Hsp90-client protein interactions (Ali et al., 2006; McLaughlin et al., 2006; Morishima et al., 2003). In this context, co- p23 is known to mark steroid receptors in the apo-state that are poised to bind hormone (Morishima et al., 2003). These data imply that in prostate cancer cells, FKBP51 levels may be limiting for the formation of p23-Hsp90-client protein complexes.

PARAGRAPH 7. *FKBP51 promotes p23 binding to Hsp90.* Our data suggested that FKBP51 might control AR activity by regulating chaperone complex assembly. Such a pathway could help define the number of AR molecules that attain the conformation required for the LBD to bind androgen. To more clearly define the contribution of FKBP51 in this pathway, we used recombinant proteins to test whether FKBP51 regulates chaperone complex assembly in a purified system. Hsp90, p23, and FKBP51 (1 uM each) were incubated alone and in various combinations. The samples were then subjected to gel filtration chromatography, and the eluted fractions were examined by immunoblotting. In this assay, protein assembly into the chaperone complex results in co-elution with Hsp90. We found that p23 interacted weakly with Hsp90 (FIG. 5B, lanes 3,4 upper panels, Appendix), but the interaction was enhanced markedly by the addition of FKBP51 and resulted in formation an FKBP51-p23-Hsp90 ternary complex (FIG. 5B, lanes 3, 4, lower panels, Appendix, part of TASK 15).

PARAGRAPH 8. Using the gel filtration assay, we examined the nucleotide requirements and effects of inhibitors on FKBP51-p23-Hsp90 ternary complex formation *in vitro*. Nucleotides and inhibitors were added to the assembly reactions prior to chromatography. The amount of p23 co-eluting with Hsp90 was quantified and plotted as histograms. Assembly was supported by ATP or AMP-PNP, indicating a requirement for ATP binding to Hsp90 but not hydrolysis. Moreover, ternary complex assembly could be inhibited by geldanomycin (GA) or FK506, drugs that bind to Hsp90 and FKBP51, respectively (FIG. 5C, Appendix). We corroborated the results of our chaperone assembly assays (with recombinant proteins) by isolating chaperone complexes from cultured cells. Following a brief (1 hr) treatment of cells with GA and FK506, FKBP51 complexes were isolated by IP and the chaperone content was analyzed by immunoblotting. Treatment with GA essentially abolished p23 recruitment into the FKBP51-Hsp90 complex, though it did not appear to affect Hsp90 binding to FKBP51 (FIG. 5D, lane 3, Appendix). FK506 reduced the levels of both Hsp90 and p23 associate with FKBP51 (FIG. 5D, lane 4, Appendix). Our data suggests that Hsp90 mediates the interaction between FKBP51 and p23. We tested this using recombinant proteins and gel filtration chromatography. When Hsp90 is omitted from the binding reaction, FKBP51 and p23 do not form a complex (FIG. 5E, Appendix). These data suggest that FKBP51 modulates p23 binding to Hsp90 through its contact with Hsp90 and not by direct contact with p23.

PARAGRAPH 9. *FKBP51 increases the number of androgen-bound AR molecules in the cell.* Since Hsp90 binds the LBD of steroid hormone receptors (Pratt and Toft, 1997a) and FKBP51 promotes Hsp90 ternary complex assembly, we anticipated that FKBP51 would regulate the LBD of AR. We tested this by deleting the LBD of AR and analyzing the chaperone content of AR isolated by IP. Neither FKBP51 nor p23 were detected in AR complexes when the LBD was deleted from AR (FIG. 6, Appendix). Our observations together with the established role of Hsp90 in the steroid hormone binding cycle led us to hypothesize that FKBP51 promotes a step in the AR ligand binding cycle. To formally address whether FKBP51 affects androgen binding to AR, we transfected AR into AR-negative PC-3 cells without and with FKBP51 and performed whole-cell hormone binding assays. Remarkably, FKBP51 expression increased the number of androgen binding sites by 38% without changing AR levels (FIG. 7A, Appendix). Moreover, FKBP51 increased androgen binding in the whole cell assay without increasing the affinity of AR for androgen (FIG. 7A).

PARAGRAPH 10. To establish that the increase in androgen binding in the whole cell assay was due to androgen binding directly to AR (as opposed to a cellular factor whose expression was induced by AR), we used a quantitative, co-IP assay developed in our laboratory (Yang et al., 2007) that discriminates between androgen-free and androgen-bound forms of AR. In the presence of SV40 small t antigen, protein phosphatase 2A (PP2A) undergoes highly selective loading onto androgen-bound AR (Yang et al., 2007). Using the PP2A-AR interaction assay, we found that FKBP51 expression approximately doubled the ratio of PP2A to AR in an IP from cells treated with androgen. Treating cells with FK506 blocked the stimulatory effect of FKBP51 in this assay, but it did not reduce the basal level of androgen binding to AR (FIG. 7B, Appendix). This result suggests that even in the absence of FKBP51 activity, a pool of AR molecules acquire competence for androgen binding. While neither FKBP51 nor FKBP52 is

required for the androgen binding cycle of AR, the expression of these co-chaperones is critical for physiological levels of AR signaling during development and in prostate cancer. In summary, our data shows that FKBP51 promotes assembly of a superchaperone-AR complex marked by p23, and this leads to a substantial increase in the number of androgen-bound AR molecules in the cell. FKBP51 over-expression therefore provides a pathway for increasing AR signaling, which is known to promote in AI-type growth in human prostate cancer models (Chen et al., 2004).

PARAGRAPH 11. *FKBP51 activity towards AR is dependent on prolyl isomerase activity and Hsp90 binding.* We used FKBP51 mutants to determine whether prolyl isomerase activity and Hsp90 binding is important for FKBP51 stimulation of AR activity on the PSA promoter. The M1 mutant characterized by Smith and co-workers (Barent et al., 1998) has two amino acid changes in the FK1 domain that reduce prolyl isomerase activity. Mutations in the TPR domain that disrupt binding to Hsp90, described originally by Chinkers and associates in PP5 (Russell et al., 1999), were also constructed (M2). FKBP51 enhancement of AR activity measured on the PSA promoter was impaired by the M1 and M2 mutations, which in combination (M1/M2) gave a larger reduction in FKBP51 activity towards AR (FIG. 8B, Appendix). The prolyl isomerase mutation (M1) reduced the ability of FKBP51 to promote p23 assembly into the Hsp90 chaperone complex without affecting FKBP51 binding to Hsp90 (FIG. 8B, Appendix). Finally, the M1, M2, and M1/M2 mutants have reduced activity in the PP2A-AR interaction assay, which is a readout of the number of AR molecules that are bound with androgen (FIG. 8C, Appendix). Our data indicates physical contact with Hsp90 (via the TPR domain) and the prolyl isomerase activity (in the FK1 domain) are important for chaperone complex assembly and enhancement of AR activity.

PARAGRAPH 12. *17-AAG and FK506 inhibit androgen-stimulated cell growth.*
Inhibitors to Hsp90 are promising therapeutics for cancer because Hsp90 regulates client proteins critical for signal transduction and cell growth (Pearl et al., 2008). The Hsp90 inhibitor 17-AAG is a GA derivative with reduced hepatotoxicity in stage II clinical trials for cancers, including prostate cancer (Workman, 2004). 17-AAG and the immunophilin ligand FK506 each can inhibit prostate cancer cell growth in culture (Periyasamy et al., 2007; Saporita et al., 2007). Given our finding that *in vitro* assembly of the Hsp90-p23-FKBP51 superchaperone complex was prevented with inhibitors to Hsp90 and FKBP51 (FIG. 5D), we tested whether chaperone inhibitors were effective in blocking prostate cancer cell growth in response to androgen. 17-AAG reduced androgen-stimulated growth of FKBP51 over-expressing and control cells to a similar extent, showing that Hsp90 inhibition is sufficient to overcome the growth benefit provided by FKBP51 (FIG. 9A, B, Appendix). Treating cells with a combination of 17-AAG (5 nM) and FK506 (1mM) resulted in an additive effect on growth inhibition in both the control and FKBP51 over-expressing cells (FIG. 9A, B, Appendix, **TASK 17**). Other groups have shown that high concentrations of 17-AAG inhibit AR by affecting protein stability (Saporita et al., 2007), however, AR levels did not change significantly when cells were maintained in a low concentration of 17-AAG (10 nM) and FK506 (10 mM) for four days. CDK4, an established client of Hsp90, also showed minimal change in response to the 17-AAG and FK506 treatment (Fig. 9C, Appendix). Our data suggest that a drug combination that targets the Hsp90 super-chaperone complex can effectively inhibit androgen-stimulated growth of prostate cancer cells without causing major changes in CDK4 and Hsp70 levels.

PARAGRAPH 13. *Additional Activities Pertinent to the Statement of Work (SOW)*
Our analysis of FKBP51 expression using prostate cancer tissue microarrays (TMAs) was initiated using “in-house” slides, and material from the NCI Cooperative Prostate Cancer Tissue Resource (CPCTR). We are still optimizing the antibodies using samples from cultured cells and our in-house TMA (**TASK1, 2**) to ensure there is no cross-reactivity with the closely related co-chaperone FKBP52. Unfortunately the CPCTR no longer provides TMAs (due to lack of NCI funding). To address this need, I have located a collaborator (Dr. Scott Lucia) at the University of Colorado who should be able to perform the IHC on his in-house prostate cancer TMAs. Thus, **TASK 3** has not been initiated, so **TASK 4** has not been initiated, because it consists of statistically analysis of data generated by **TASK 3**.

PARAGRAPH 14. We have developed multiple prostate cancer cell lines that over-express FKBP51: LNCaP, C4-2, C4-2b, CWR22rv1, and PC-3. We also developed stable lines that express shRNA to FKBP51, resulting in reduced levels of FKBP51 expression (10-30% of controls). Characterization of these cells lines (**TASK 5**) has been initiated. Characterization of these cell lines needs to be completed before we can move on to more complex analysis with xenografts (**TASK 6)** and bone injection experiments (**TASK 7)** including whether cell growth at different organ sites is androgen-dependent **(TASK 10**). Thus, **TASKS 6-8** have not been initiated. The reason we developed additional lines is because sub-cutaneous tumor growth rates of our pre-existing cell lines were very slow (**TASK 6**), and we were concerned whether the pre-existing lines would show sufficient growth in bone experiments, which are very costly and labor intensive (**TASK 9, 10**). Since **TASK 11** involves repeating and refining experiments, it has not been initiated.

KEY RESEARCH ACCOMPLISHMENTS:

1. FKBP51 is over-expressed in a xenograft model of AI prostate cancer at both the mRNA and protein levels.

2. FKBP51 Positively Regulates AR activity towards both reporter genes and endogenous genes, and that FKBP51 alters AR responsiveness to different ligands.

3. FKBP51 expression alters the chaperone composition of AR complexes, and that FKBP51 increases the number of androgen-bound AR molecules in the cell.

4. FKBP51 activity towards AR is dependent on prolyl isomerase activity and Hsp90 binding.

5. Inhibitors to Hsp90 (17-AAG) and FKBP51 (FK506) inhibit androgen stimulated cell growth, and can be used in combination at low concentrations to avoid induction of Hsp70.

REPORTABLE OUTCOMES:

FKBP51 promotes assembly of the Hsp90 chaperone complex and regulates androgen receptor signaling in prostate cancer cells.

Ni L, Yang CS, Gioeli D, Frierson H, Toft DO, Paschal BM.
Mol Cell Biol. 2010 (5):1243-53. PMID: 20048054 [PubMed - indexed for MEDLINE]

CONCLUSION:

We took a highly focused approach that involved testing whether the expression of protein chaperones with known links to steroid hormone binding are altered in AI prostate cancer, and found that the co-chaperone FKBP51 is over-expressed in AI xenografts propagated in castrated mice. FKBP51 is a peptidyl prolyl isomerase that binds the immunosuppressants FK506 and rapamycin.
We found that FKBP51 promotes AR-dependent transcription using both reporter- and endogenous gene-based assays, consistent with published data (Febbo et al., 2005). Moreover, FKBP51 enhanced LNCaP cell growth in response to low (50 pM) androgen. The growth benefit of FKBP51 in LNCaP cells was inhibited by 17-AAG, which links FKBP51 to Hsp90 function. We obtained direct evidence that FKBP51 induces assembly of the Hsp90 superchaperone complex, and that this regulates the LBD of AR. This involves FKBP51 contact with Hsp90 and recruitment of p23, a co-chaperone known to stabilize Hsp90 binding to client proteins (McLaughlin et al., 2006). Crystallographic analysis revealed that p23 recognizes the ATP-bound, closed conformation of Hsp90; a major contact surface for p23 is formed by “lid” closure of Hsp90 (Ali et al., 2006). Thus, FKBP51 may promote ATP binding or lid closure, or it may stabilize another mobile element in Hsp90 structure such as the catalytic loop that is contacted by p23 (Ali et al., 2006). Hsp90 inhibitors are promising as therapeutics because Hsp90 regulates client proteins

including AR that are critical for signal transduction and cell growth in cancer (Pearl et al., 2008). High dose 17-AAG, however, causes skeletal complications and increases Hsp70 levels, the latter of which may promote tumor resistance to therapy (Powers et al., 2008; Price et al., 2005). Hsp90 inhibitors used in combination with drugs that target co-chaperones that function in the same multi-subunit complex is a potential strategy for inhibiting clients such as AR that are highly dependent on the superchaperone complex. By promoting Hsp90 superchaperone complex assembly, FKBP51 has a quantitative effect on androgen signaling by increasing the number of AR molecules that are subsequently loaded with hormone. Because androgen-bound AR regulates FKBP51 expression, this creates a feed-forward mechanism that could amplify AR signaling under low hormone conditions that occur during androgen ablation. Our studies validate FKBP51 as a drug target in advanced prostate cancer.

REFERENCES:

Ali, M. M., Roe, S. M., Vaughan, C. K., Meyer, P., Panaretou, B., Piper, P. W., Prodromou, C., and Pearl, L. H. (2006). Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature *440*, 1013-1017.

Barent, R. L., Nair, S. C., Carr, D. C., Ruan, Y., Rimerman, R. A., Fulton, J., Zhang, Y., and Smith, D. F. (1998). Analysis of FKBP51/FKBP52 chimeras and mutants for Hsp90 binding and association with progesterone receptor complexes. Mol Endocrinol *12*, 342-354.

Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004). Molecular determinants of resistance to antiandrogen therapy.[see comment]. Nature Medicine *10*, 33-39.

Cheung-Flynn, J., Prapapanich, V., Cox, M. B., Riggs, D. L., Suarez-Quian, C., and Smith, D. F. (2005). Physiological role for the cochaperone FKBP52 in androgen receptor signaling. Mol Endocrinol *19*, 1654-1666.

Denny, W. B., Valentine, D. L., Reynolds, P. D., Smith, D. F., and Scammell, J. G. (2000). Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology *141*, 4107-4113.

Febbo, P. G., Lowenberg, M., Thorner, A. R., Brown, M., Loda, M., and Golub, T. R. (2005). Androgen mediated regulation and functional implications of fkbp51 expression in prostate cancer.[see comment]. Journal of Urology *173*, 1772-1777.

Kesler, C. T., Gioeli, D., Conaway, M. R., Weber, M. J., and Paschal, B. M. (2007). Subcellular localization modulates activation function 1 domain phosphorylation in the androgen receptor. Mol Endocrinol *21*, 2071-2084.

Klein, K. A., Reiter, R. E., Redula, J., Moradi, H., Zhu, X. L., Brothman, A. R., Lamb, D. J., Marcelli, M., Belldegrun, A., Witte, O. N., and Sawyers, C. L. (1997). Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nature Medicine *3*, 402-408.

Magee, J. A., Chang, L. W., Stormo, G. D., and Milbrandt, J. (2006). Direct, androgen receptor-mediated regulation of the FKBP5 gene via a distal enhancer element. Endocrinology *147*, 590-598.

McLaughlin, S. H., Sobott, F., Yao, Z. P., Zhang, W., Nielsen, P. R., Grossmann, J. G., Laue, E. D., Robinson, C. V., and Jackson, S. E. (2006). The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. J Mol Biol *356*, 746-758.

Morishima, Y., Kanelakis, K. C., Murphy, P. J., Lowe, E. R., Jenkins, G. J., Osawa, Y., Sunahara, R. K., and Pratt, W. B. (2003). The hsp90 cochaperone p23 is the limiting component of the multiprotein hsp90/hsp70-based chaperone system in vivo where it acts to stabilize the client protein: hsp90 complex. J Biol Chem *278*, 48754-48763.

Nagabhushan, M., Miller, C. M., Pretlow, T. P., Giaconia, J. M., Edgehouse, N. L., Schwartz, S., Kung, H. J., de Vere White, R. W., Gumerlock, P. H., Resnick, M. I.*, et al.* (1996). CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res *56*, 3042-3046.

Pearl, L. H., Prodromou, C., and Workman, P. (2008). The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem J *410*, 439-453.

Periyasamy, S., Warrier, M., Tillekeratne, M. P., Shou, W., and Sanchez, E. R. (2007). The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and -independent mechanisms. Endocrinology *148*, 4716-4726.

Plymate, S. R., Tennant, M. K., Culp, S. H., Woodke, L., Marcelli, M., Colman, I., Nelson, P. S., Carroll, J. M., Roberts, C. T. J., and Ware, J. L. (2004). Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. Prostate *61*, 276-290.

Powers, M. V., Clarke, P. A., and Workman, P. (2008). Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. Cancer Cell *14*, 250-262.

Pratt, W. B., and Toft, D. (1997a). Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones. Endocrine Reviews *18*, 306-360.

Pratt, W. B., and Toft, D. (1997b). Steroid Receptor Interactions with Heat Shock Protein and Immunophilin Chaperones. Endocrine Rev *18*, 306-360.

Price, J. T., Quinn, J. M. W., Sims, N. A., Vieusseux, J., Waldeck, K., Docherty, S. E., Myers, D., Nakamura, A., Waltham, M. C., Gillespie, M. T., and Thompson, E. W.

(2005). The heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, enhances osteoclast formation and potentiates bone metastasis of a human breast cancer cell line. Cancer Research *65*, 4929-4938.

Ratajczak, T., Ward, B. K., and Minchin, R. F. (2003). Immunophilin chaperones in steroid receptor signalling. Curr Top Med Chem *3*, 1348-1357.

Riggs, D. L., Cox, M. B., Tardif, H. L., Hessling, M., Buchner, J., and Smith, D. F. (2007). Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. Mol Cell Biol *27*, 8658-8669.

Riggs, D. L., Roberts, P. J., Chirillo, S. C., Cheung-Flynn, J., Prapapanich, V., Ratajczak, T., Gaber, R., Picard, D., and Smith, D. F. (2003). The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. EMBO J *22*, 1158-1167.

Russell, L. C., Whitt, S. R., Chen, M. S., and Chinkers, M. (1999). Identification of conserved residues required for the binding of a tetratricopeptide repeat domain to heat shock protein 90. J Biol Chem *274*, 20060-20063.

Saporita, A. J., Ai, J., and Wang, Z. (2007). The Hsp90 inhibitor, 17-AAG, prevents the ligand-independent nuclear localization of androgen receptor in refractory prostate cancer cells. Prostate *67*, 509-520.

Workman, P. (2004). Altered states: selectively drugging the Hsp90 cancer chaperone. Trends Mol Med *10*, 47-51.

Yang, C.-S., Xin, H.-W., Kelley, J. B., Spencer, A., Brautigan, D. L., and Paschal, B. M. (2007). Ligand binding to the androgen receptor induces conformational changes that regulate phosphatase interactions. Molecular & Cellular Biology *27*, 3390-3404.

Yeh, W. C., Li, T. K., Bierer, B. E., and McKnight, S. L. (1995). Identification and characterization of an immunophilin expressed during the clonal expansion phase of adipocyte differentiation. Proc Natl Acad Sci U S A *92*, 11081-11085.

Yong, W., Yang, Z., Periyasamy, S., Chen, H., Yucel, S., Li, W., Lin, L. Y., Wolf, I. M., Cohn, M. J., Baskin, L. S*., et al.* (2007a). Essential role for Co-chaperone Fkbp52 but not Fkbp51 in androgen receptor-mediated signaling and physiology. J Biol Chem *282*, 5026-5036.

Yong, W., Yang, Z., Periyasamy, S., Chen, H., Yucel, S., Li, W., Lin, L. Y., Wolf, I. M., Cohn, M. J., Baskin, L. S*., et al.* (2007b). Essential role for Co-chaperone Fkbp52 but not Fkbp51 in androgen receptor-mediated signaling and physiology. J Biol Chem *282*, 5026-5036.

APPENDIX:

pdf of Li et al is attached

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08
zjs / DOCHEAD=ARTICLE zjss / DOCTOPIC=ARTICLES
FROM COVER SHEET: Editor: Neel Article No.: T Section: Articles

MOLECULAR AND CELLULAR BIOLOGY, Mar. 2010, p. 000 Vol. 30, No. 5
0270-7306/10/$12.00 doi:10.1128/MCB.01891-08

FKBP51 Promotes Assembly of the Hsp90 Chaperone Complex and Regulates Androgen Receptor Signaling in Prostate Cancer Cells[▽]

Li Ni,[1] Chun-Song Yang,[1] Daniel Gioeli,[2] Henry Frierson,[3] David O. Toft,[4] and Bryce M. Paschal[1,5]*

AQ: A *Center for Cell Signaling[1] and Departments of Microbiology[2] and Pathology,[3] University of Virginia School of Medicine, Charlottesville, Virginia 22908; Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905[4]; and Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia 22908[5]*

Received 12 December 2008/Returned for modification 26 February 2009/Accepted 23 December 2009

Prostate cancer progression to the androgen-independent (AI) state involves acquisition of pathways that allow tumor growth under low-androgen conditions. We hypothesized that expression of molecular chaperones that modulate androgen binding to AR might be altered in prostate cancer and contribute to progression to the AI state. Here, we report that the Hsp90 cochaperone FKBP51 is upregulated in LAPC-4 AI tumors grown in castrated mice and describe a molecular mechanism by which FKBP51 regulates AR activity. Using recombinant proteins, we show that FKBP51 stimulates recruitment of the cochaperone p23 to the ATP-bound form of Hsp90, forming an FKBP51-Hsp90-p23 superchaperone complex. In cells, FKBP51 expression promotes superchaperone complex association with AR and increases the number of AR molecules that undergo androgen binding. FKBP51 stimulates androgen-dependent transcription and cell growth, and FKBP51 is part of a positive feedback loop that is regulated by AR and androgen. Finally, depleting FKBP51 levels by short hairpin RNA reduces the transcript levels of genes regulated by AR and androgen. Because the superchaperone complex plays a critical role in determining the ligand-binding competence and transcription function of AR, it provides an attractive target for inhibiting AR activity in prostate cancer cells.

Fn1 Defining the etiology of prostate cancer remains an area of active investigation, but there is a growing focus on the role of the androgen receptor (AR) in the context of "androgen-independent" (AI) signaling during disease progression (5). Like other members of the nuclear receptor superfamily, AR is a transcription factor that regulates the expression of scores of genes important for cell growth and development. Nuclear receptors have a conserved domain organization that includes an N-terminal AF-1 domain, a central DNA-binding domain, a hinge region, and a C-terminal ligand-binding domain (LBD). AR activity is normally regulated by androgen binding to the LBD, an event that initiates changes in AR conformation, subcellular localization, and interactions with cofactors that facilitate transcription from target genes (31). AI prostate cancers can grow in a low-androgen environment, which occurs in the setting of androgen ablation therapy in patients and surgical castration in animal models.

Ligand binding to nuclear receptors, including AR, is regulated by molecular chaperones. The central player in this reaction is Hsp90, an ATP-utilizing chaperone that interacts transiently with LBDs to stabilize a conformation that is appropriate for ligand binding (24). Hsp90 operates as a molecular chaperone for numerous client proteins by cycling through conformational changes coupled to ATP binding and hydrolysis (19). Additionally, accessory proteins termed cochaperones facilitate or stabilize changes in Hsp90 conformation and ATPase activity. One well-studied cochaperone originally identified in progesterone receptor (PR) complexes is p23 (15). Through selective binding to the ATP form of Hsp90, p23 stabilizes the "closed" state and promotes Hsp90 interactions with client proteins (14, 33). p23 function is critical for steroid hormone receptors, as it has been shown to act as a limiting component for assembly of the multicomponent Hsp90 "superchaperone" complex that stabilizes the LBD in a conformation that is competent for hormone binding (15). p23 function has also been linked to later steps of nuclear receptor function, including transcription complex disassembly (8).

Less well understood are the cochaperones that belong to the immunophilin class of proteins, FK506-binding proteins FKBP51 and FKBP52, and cyclosporine A-binding protein Cyp40. These cochaperones have N-terminal domains with peptidyl-prolyl isomerase activity and C-terminal domains that contain three tetratricopeptide repeats (TPR) that mediate binding to Hsp90 (26). The functions of FKBP51 and FKBP52, which are 70% identical at the protein level, have been studied mostly in the context of the PR and the glucocorticoid receptor (GR). FKBP51 negatively regulates GR and PR activity by reducing hormone binding affinity (6, 9). In contrast, FKBP52 enhances GR, PR, and AR responsiveness to cognate hormone (28). FKBP52 knockout mice have developmental defects in reproductive tissues (in males) consistent with reduced AR signaling and a failure of embryo implantation (in females) consistent with reduced PR signaling (4, 37). These observations, together with the apparent absence of a phenotype in mice lacking FKBP51, led to the conclusion that FKBP51 does not play a significant role in AR signaling (37).

AR overexpression is a signature of AI disease, and forcing AR expression in model systems is sufficient to generate AI growth and responsiveness to antiandrogens (3). Assuming

* Corresponding author. Mailing address: Box 800577 Health Systems, Room 7021 West Complex, Center for Cell Signaling, University of Virginia, Charlottesville, VA 22908. Phone: (434) 243-6521. Fax: (434) 924-1236. E-mail: paschal@virginia.edu.

▽ Published ahead of print on 4 January 2010.

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

that AR activity is a key determinant of disease progression, it stands to reason that tumor cells might employ additional mechanisms to increase AR activity. Based on this rationale, we examined whether chaperones known to regulate the ligand-binding cycle of AR are altered during prostate cancer progression and promote AI or androgen-hypersensitive growth. Here we show the Hsp90 cochaperone FKBP51 is overexpressed in a xenograft model of AI prostate cancer and describe a molecular mechanism by which FKBP51 promotes AR signaling in prostate cancer cells. Published microarray data have shown that FKBP51 message levels are higher in metastatic tumors than in tumors confined to the prostate (7). Thus, FKBP51 might be exploited as a drug target for inhibiting AR function in certain advanced prostate cancers.

MATERIALS AND METHODS

Xenografts and cell lines. LAPC-4 tumor cells (3×10^5) were combined 1:1 with Matrigel and injected subcutaneously into the flanks of 6-week-old intact or castrated *scid* mice as described previously (3). Xenograft tumors were harvested upon reaching ~1 cm^3. For androgen-dependent (AD) and AI tumors, this required approximately 8 and 18 weeks of growth in *scid* mice, respectively. Tumors were flash frozen, ground under liquid N_2 using a mortar and pestle, resuspended in 5 volumes of lysis buffer (50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl,1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol [DTT], and 1 μg/ml each aprotinin, leupeptin, and pepstatin), disrupted with a Polytron, and clarified by centrifugation (30,000 × *g*, 30 min). Tumor extracts (5 mg/ml) were diluted with polyacrylamide gel sample buffer and analyzed by immunoblotting. Prostate cancer cell lines (PC-3, AR negative; LNCaP and CWR22Rv1, AR positive) were obtained from the American Type Culture Collection and grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS). Twenty-four hours before ligand addition, cells were transferred to phenol red-free medium containing charcoal- and dextran-treated FBS.

Antibodies. The following antibodies were used for immunoblotting at a dilution of 1:1,000 unless noted otherwise: Hsp90 (H90-10) (2), Hsp70 (BB70) (32), p23 (JJ3) (10), FKBP51 (Hi51B), FKBP52 (Hi52A) (18), PP2Ac (ID6; Upstate), AR (AR21, 1:5,000) (35), and PSA (AR014-5RE, 1:100; Biogenix). FKBP51 (clone 18, 1:20; BD Transduction) was used for immunocytochemistry.

Immunocytochemistry. Xenografts were fixed in zinc formalin, dehydrated, and embedded in paraffin. Sections were cut, mounted on glass slides, rehydrated in citrate buffer, and microwaved for antigen recovery. FKBP51 monoclonal antibody clone 18 was used along with biotinylated secondary antibody, avidin-peroxidase, and diaminobenzidine as the chromogen.

Lentivirus. LNCaP cells stably expressing Flag-tagged human FKBP51 were prepared using lentivirus generated by standard methods (pLenti4/TO/V5-DEST, psPAX, and pMD2G plasmids) in 293T cells. Cell culture medium from 293T cells containing the lentivirus was used to infect LNCaP cells, with subsequent selection in the presence of Zeocin (Invitrogen). The LacZ control line was generated by similar methods with a plasmid that encodes β-galactosidase (pLenti4/TO/V5-GW/lacZ). FKBP51 knockdown lines of LNCaP and CWR22Rv1 cells were prepared using pLKO.1-puro plasmids (Sigma), and green fluorescent protein (GFP) short hairpin RNA (shRNA) was used as the control.

Transcription assays. AR transcription was measured using the dual-luciferase reporter system and plotted as firefly/*Renilla* as described previously (35). The firefly luciferase reporter genes used to measure AR activity were based on the promoters for prostate-specific antigen (PSA), probasin, mouse mammary tumor virus (MMTV), and synthetic androgen response elements (AREs). Reactions were prepared in triplicate, the results were analyzed using the Student *t* test, and the data presented are representative of at least three experiments. Transcript levels of endogenous AR-regulated genes (*KLK2*, *PSA*, *S100P*, *TMPRSS2*, and *FKBP51*) were measured by real-time PCR. Reactions were prepared in duplicate, the results were analyzed by using the Student *t* test, and the data are representative of at least two experiments. In brief, cells were treated with ligands for 24 h and RNA was prepared by using an RNeasy mini kit (Qiagen) including a DNase treatment step. Following quantitation with Ribogreen (Molecular Probes), 1 μg of RNA was reverse transcribed using iScript and amplified using the IQ SYBR green PCR master mix in a MyiQ instrument (all from Bio-Rad). The relative mRNA level was normalized to the β-glucuronidase (*GUS*) mRNA level in the same sample by the $2^{-\Delta\Delta CT}$ method. The forward and reverse primers used for real-time PCR were as follows: *KLK2*, 5′-CACAGCTGCCCATTGCCTAAAGAA-3′ and 5′-GGCCTGTGTCTTCAG GCTCAAA-3′; *PSA*, 5′-TGGTGCATTACCGGAAAGTGGATCA-3′ and 5′-G CTTGAGTCTTGGCCTGGTCATTTC-3′; S100P, 5′-ATGACGGAACTAGA GACAGCC-3′ and 5′-AGGAAGCCTGGTAGCTCCTT-3′; TMPRSS2, 5′-GG ACAGTGTGCACCTCAAAGAC-3′ and 5′-TCCCACGAGGAAGGTCCC-3′; FKBP51, 5′-AGGAGGGAAGAGTCCCAGTG-3′ and 5′-TGGGAAGCTACT GGTTTTGC-3′. Note that FKBP51 is encoded by the *FKBP5* gene.

Growth assays. Cell lines were seeded into 96-well dishes (3×10^3 cells/well) and grown for 24 h. Medium containing ligands or drugs was changed on days 1, 3, and 5. On the day of measurement, cells were incubated in medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 125 μg/ml for 4 h. Reduced MTT was measured using A_{595}. Samples were performed in sextuplet and plotted as the mean ± the standard deviation.

Isolation of AR and chaperone complexes. AR and chaperone complexes were isolated by immunoprecipitation (IP). PC-3 cells were cotransfected with expression plasmids including pcDNA3-Flag-AR with either PCI-FKBP51 or control plasmids. Twenty-four hours posttransfection, cells were treated with 1 nM R1881 for the indicated times and lysed in 500 μl IP buffer (20 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 20 mM Na_2MoO_4, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, protease inhibitors, and a phosphatase inhibitor cocktail [Sigma]). The lysate was sonicated and cleared by centrifugation. Samples were immunoprecipitated for 4 h at 4°C using anti-Flag M2 agarose beads (Sigma). Complexes were washed three times with IP buffer, solubilized in polyacrylamide gel sample buffer, and analyzed by electrophoresis and immunoblotting.

Chaperone complexes were assembled using purified recombinant proteins FKBP51, Hsp90, and p23 expressed in bacteria. Proteins were combined (1 μM each), incubated for 1 h at 30°C, clarified at >100,000 × *g*, and chromatographed on a Superose 12 or Superdex 75 gel filtration column (40 mM HEPES-KOH, pH 7.4, containing 50 mM KCl and 2 mM $MgCl_2$) at a flow rate of 0.5 ml/min. Column fractions (0.5 ml each) were analyzed by immunoblotting using peroxidase-labeled secondary antibodies and enhanced chemiluminescence. p23 quantitation was performed using Alexa Fluor 680-labeled secondary antibody and infrared imaging (Li-Cor Biosciences). Levels of p23 bound to hsp90 were determined by the levels of p23 in fractions 1 to 6 versus the total p23 levels in fractions 1 to 12.

Androgen binding assays. A whole-cell binding assay with [^{3}H]R1881 was performed with transfected PC-3 cells. pcDNA3-Flag-AR was cotransfected with PCI-FKBP51 or PCI-Neo into PC-3 cells. Cells were treated with the indicated concentrations of [^{3}H]R1881 for 1 h and washed with phosphate-buffered saline, and the total bound [^{3}H]R1881 was extracted in cold ethanol and detected in a scintillation counter. Nonspecific binding was measured at each [^{3}H]R1881 concentration by the addition of 1 μM unlabeled R1881 and then subtracted from the total binding. Nonlinear regression (OriginPro 7.5) was used to determine the B_{max} and K_d using the following equation: Specific binding = B_{max} [L]/K_d + [*L*], where [*L*] is the concentration of [^{3}H]R1881.

Changes in the number of AR molecules that undergo androgen binding in response to FKBP51 expression were also assessed by using the AR-PP2A interaction, which is strictly dependent on the agonist conformation of AR (35). PC-3 cells were cotransfected with expression plasmids including pcDNA3-Flag-AR and simian virus 40 (SV40) small t antigen with either PCI-FKBP51 or a control plasmid. Twenty-four hours posttransfection, cells were pretreated with 10 μM FK506 for 1 h (where indicated) and then treated with 1 nM R1881 for 1 h. The cells were lysed in 500 μl IP buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, and protease inhibitors. The lysate was then sonicated and cleared by centrifugation. AR complexes were immunoprecipitated and washed as described above. Levels of transfected AR and endogenous PP2Ac in the complex were quantified by immunoblotting using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

RESULTS

FKBP51 is overexpressed in a xenograft model of AI prostate cancer. We reasoned that chaperones involved in regulating the ligand-binding cycle of AR might be altered during prostate cancer progression and promote AI or androgen-hypersensitive growth. We tested this hypothesis by comparing expression levels of chaperones in LAPC-4 xenografts propagated in *scid* mice (12). LAPC-4 AD and AI cells were grown

AQ: B

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

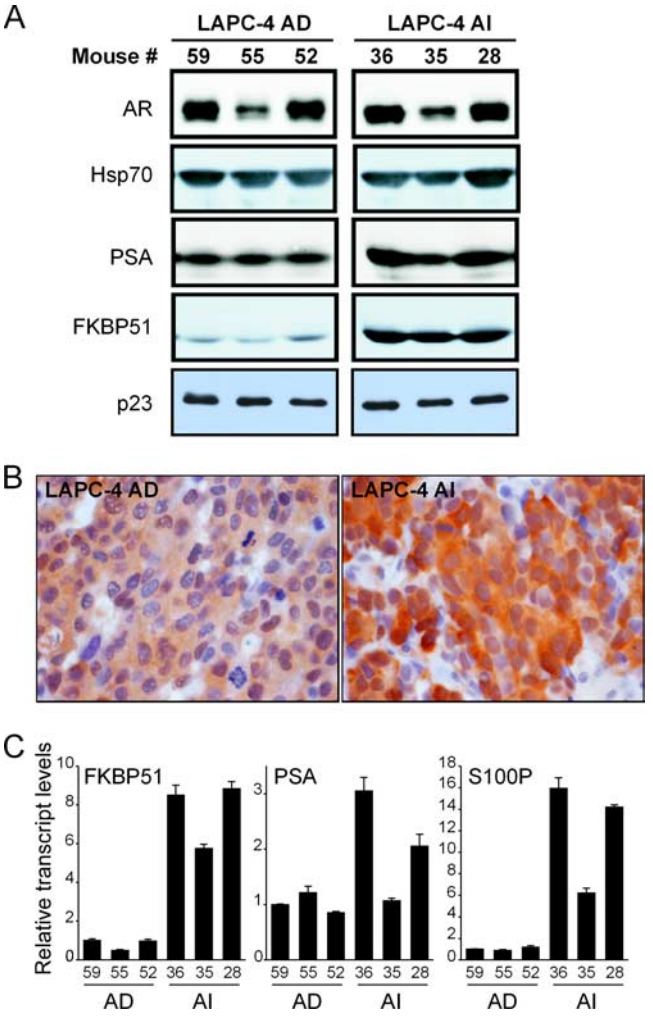


FIG. 1. FKBP51 is overexpressed in a xenograft model of AI prostate cancer. (A) Immunoblotting for chaperones in LAPC-4 AD and AI tumors grown in intact and castrated *scid* mice, respectively. The AD and AI tumors were propagated and harvested as described in Materials and Methods. The mouse number (#) refers to the particular animal from which the tumor was harvested. (B) Immunocytochemistry of AD and AI tumors using an FKBP51 monoclonal antibody. (C) Real-time PCR to determine *FKBP51*, *PSA*, and *S100P* transcript levels in AD and AI tumors. *FKBP51* overexpression was not detected in a previous microarray analysis of the LAPC-4 xenografts (3).

4 COLOR

in intact and castrated mice, respectively. By immunoblotting the tumor extracts, the levels of Hsp90, Hsp70, FKBP52, Cyp40, and p23 were found to be comparable in LAPC-4 AD and AI samples (Fig. 1A and data not shown). In contrast, FKBP51 protein was elevated about threefold in the LAPC-4 AI samples relative to that in AD samples (Fig. 1A). Immunocytochemistry with a monoclonal antibody to FKBP51 confirmed that FKBP51 overexpression occurs in the LAPC-4 AI tumors (Fig. 1B). Real-time PCR was used to show that there is a 7- to 10-fold increase in FKBP51 mRNA (Fig. 1C). PSA transcript levels, which normally are tightly regulated by AR and androgen, were slightly higher in two of three AI xenografts (Fig. 1C). Another androgen-regulated gene, *S100P*, was upregulated 6- to 15-fold in the AI xenografts (Fig. 1C). We interpreted these data as provisional evidence for a mechanism that promotes AR activity by compensating for the reduced-androgen environment of a castrated host. FKBP51 levels are also elevated in the AI xenograft generated from the AD xenograft CWR22 (7). Thus, FKBP51 is overexpressed in the two most commonly used xenograft models of AI prostate cancer.

FKBP51 positively regulates AR activity measured with reporter genes. FKBP51 is an androgen-regulated gene in prostate cancer cells (13). FKBP51 overexpression in the AI xenografts LAPC-4 and CWR22Rv1 could therefore be a result of enhanced AR signaling characteristic of the AI state. We considered the alternative hypothesis, namely, that FKBP51 overexpression enhances AR function and thereby facilitates the AI phenotype. To address this hypothesis, we tested whether FKBP51 can modulate AR activity. We assayed AR-dependent transcription in the absence or presence of cotransfected FKBP51 in the prostate cancer cell line PC-3, which is negative for both AR and FKBP51. Reintroduction of AR into PC-3 cells activates the endogenous *FKBP51* gene, an observation that is consistent with AR regulation of *FKBP51* expression (11). *FKBP51* expression resulted in a strong enhancement of androgen-stimulated transcription from a reporter gene containing the 6-kb *PSA* promoter, also consistent with a previous report (7). There was no obvious effect on AR protein levels (Fig. 2A, inset), a good indication that the FKBP51 effect on androgen-stimulated transcription is not simply a chaperone effect on AR protein stabilization. Additional evidence that FKBP51 regulates AR activity was obtained by showing that it enhances androgen-stimulated transcription from other promoters known to be regulated by AR. FKBP51 enhanced androgen-stimulated transcription measured on AREs from the probasin (22) promoter (AAR3tk-Luc; Fig. 2B) and the MMTV promoter (Fig. 2C) and on synthetic AREs (Fig. 2E). FKBP51 clearly has opposite effects on AR and GR, as FKBP51 inhibited dexamethasone (Dex)-stimulated GR transcription from the MMTV promoter (Fig. 2D) (6). FKBP51 enhancement of AR activity was inhibited by treating cells with FK506, a ligand that binds with submicromolar affinity to FKBP51 (Fig. 2F) (36). FKBP52 was also capable of enhancing AR activity in our assays, though unlike that of FKBP52, its effect was not inhibited significantly by FK506. This result is consistent with studies from Smith and coworkers who showed that FKBP52 regulation of steroid hormone receptor activity is independent of its enzyme activity (27).

Levels of FKBP51 are important for transcription of endogenous AR-regulated genes. To address the contribution of FKBP51 to endogenous gene expression, we used lentivirus to generate LNCaP cell lines that stably express Flag-FKBP51 with LacZ as a control. Flag-FKBP51 enhanced AD expression of the endogenous *PSA* gene measured by real-time PCR (Fig. 3A). Since AR and androgens are critical regulators of prostate cell growth, we examined whether FKBP51 expression promotes AD cell growth. We assayed growth of the LNCaP-FKBP51 and LNCaP-lacZ lines in the presence of androgen over 6 days (Fig. 3B). In the absence of androgen, relatively ●●●●●●●● growth of LNCaP-lacZ and LNCaP-FKBP51 cells was observed. Both lines underwent growth in response to a low concentration (50 pM) of the synthetic androgen R1881;

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

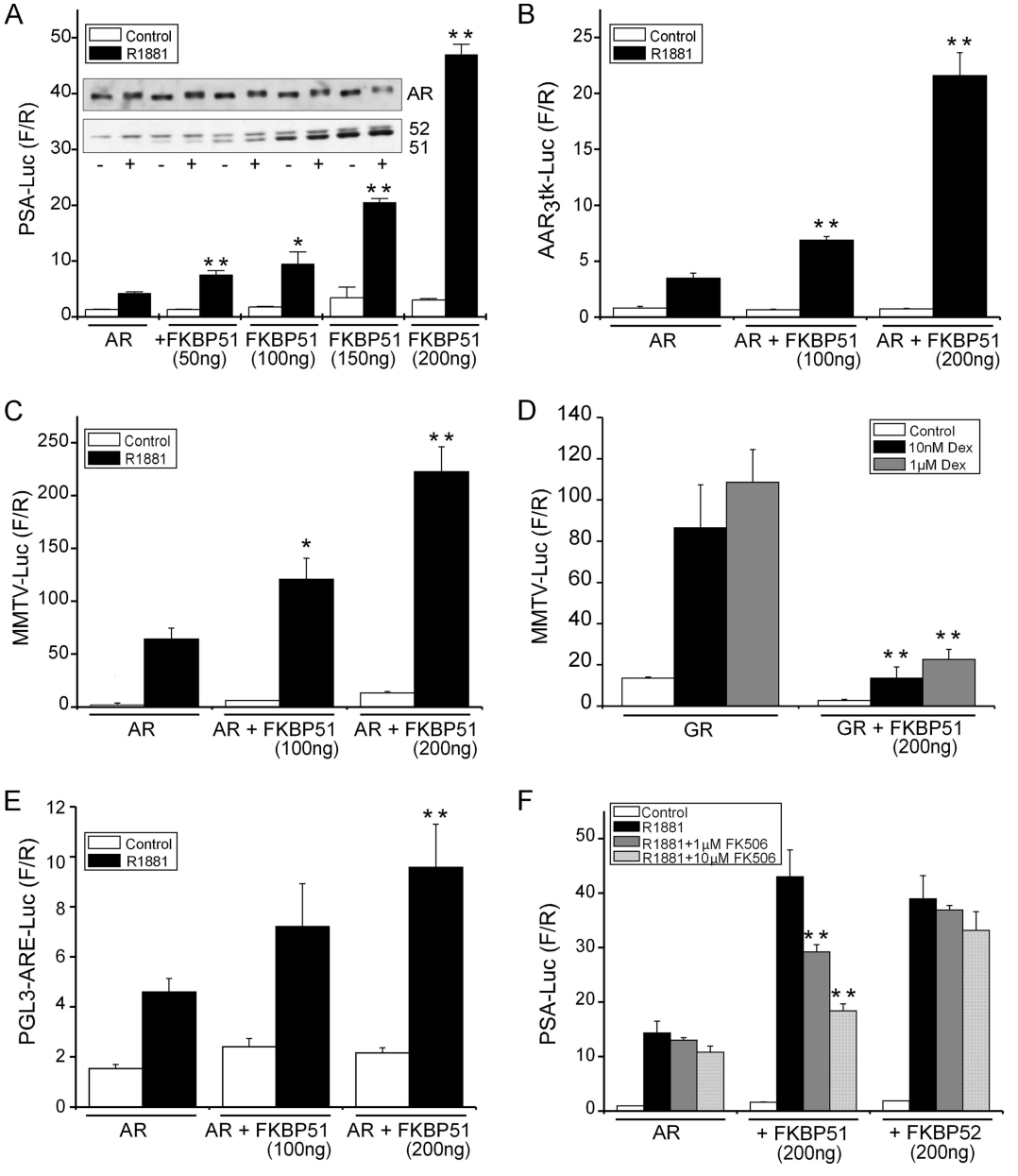


FIG. 2. FKBP51 promotes AR-dependent transcription. (A) Effect of FKBP51 on AR activity in the absence (open bars) or presence (filled bars) of 1 nM R1881. AR activity was measured by using the PSA-Luc reporter gene in PC-3 cells. AR, FKBP51, and FKBP52 levels were examined by immunoblotting (inset). FKBP51 stimulated AR activity on promoters, including the MMTV promoter, under conditions where GR activity was inhibited, as previously reported (6). (B) Effect of FKBP51 on AR activity measured by using AREs from the probasin promoter. (C) Effect of FKBP51 on AR activity using the MMTV promoter. (D) Effect of FKBP51 on GR activity using the MMTV promoter. (E) Effect of FKBP51 on AR activity using a promoter containing three synthetic AREs. (F) Effect of FK506 on FKBP51 and FKBP52 enhancement of AR activity measured by using the PSA promoter. The *P* values are denoted by asterisks (*, $P < 0.05$; **, $P < 0.01$) for effects of FKBP51 expression (A to E) and FK506 treatment (F). F/R, firefly/*Renilla*.

however, FKBP51 expression provided an advantage for cell growth in this assay ($P < 0.001$; Fig. 3B).

Because the FKBP51 knockout mouse does not display overt defects in androgen signaling (4, 37), the contribution of FKBP51 to AR function has been unclear. We considered the possibility that FKBP51 is dispensable in normal cells but that FKBP51 might be important for AR activity in prostate cancer cells. We tested whether androgen signaling through AR is dependent on endogenous FKBP51 by a knockdown approach, using lentivirus to transduce LNCaP and CWR22Rv1 cells with shRNA targeted to FKBP51. Lentivirus containing shRNA targeted to GFP was used as a control. Real-time PCR was used to measure androgen induction of AR-regulated genes in control and knockdown cell lines. In cells containing the FKBP51 shRNA, FKBP51 transcript and protein levels after androgen treatment (24 h) were reduced significantly (Fig. 3C and E). Reducing FKBP51 levels led to substantial reductions in the transcript levels for the *KLK2*, *PSA*, and *S100P* genes.

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

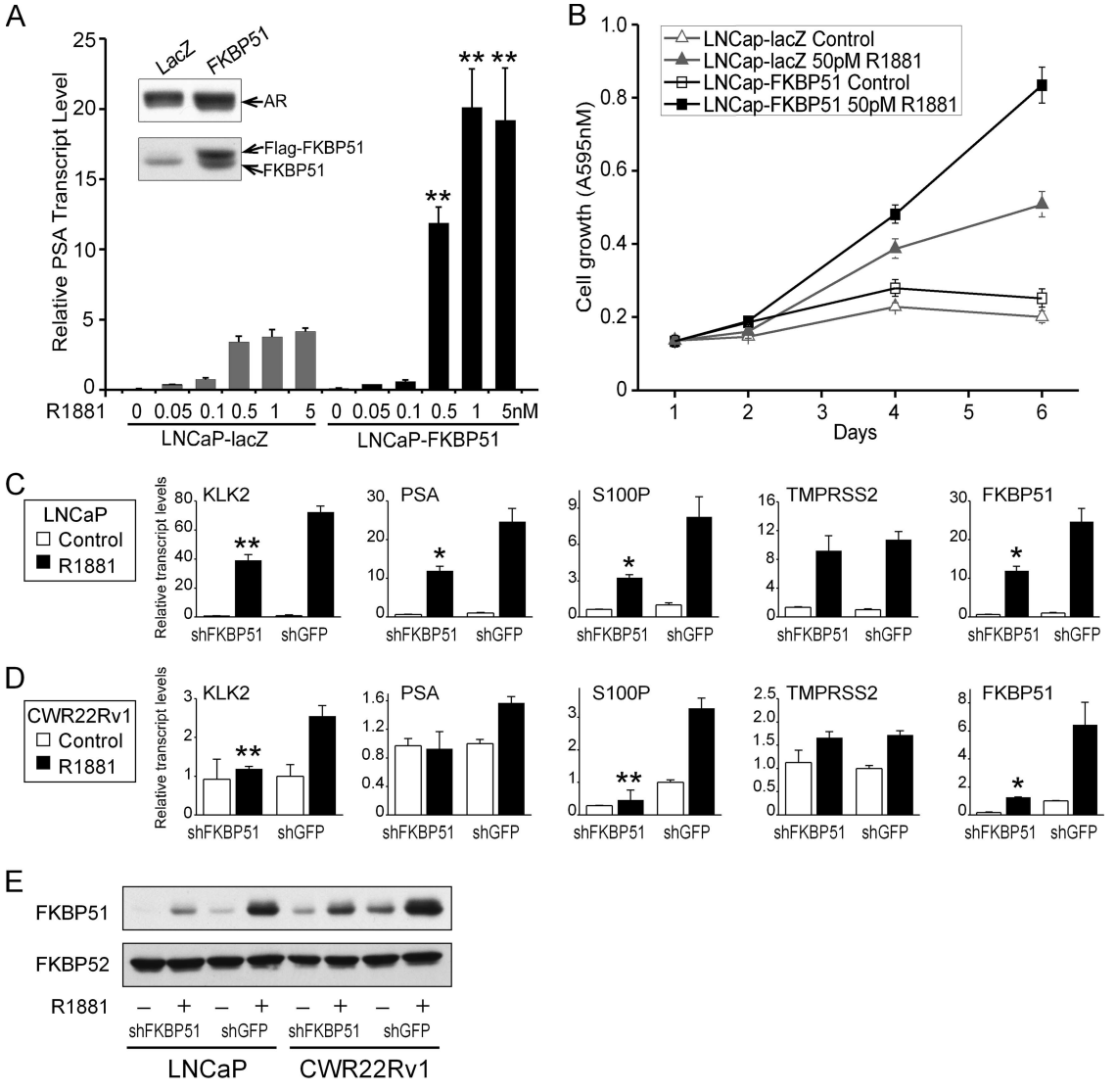


FIG. 3. Androgen-dependent transcription and cell growth in prostate cancer cells engineered for FKBP51 expression. (A) Real-time PCR measurement of endogenous PSA transcript levels in LNCaP-lacZ and LNCaP-FKBP51 cell lines treated with the indicated concentrations of synthetic androgen. (B) Androgen-stimulated LNCaP cell growth of LNCaP-lacZ and LNCaP-FKBP51 cell lines measured by the MTT assay. (C) Real-time PCR to measure transcript levels of genes regulated by androgen and AR (*KLK2*, *PSA*, *S100P*, *TMPRSS2*, *FKBP51*). (D) Real-time PCR to measure transcript levels in CWR22Rv1 cells. The *P* values are denoted by asterisks (*, $P < 0.05$; **, $P < 0.01$) for effects of FKBP51 overexpression (A) and knockdown (C and D) on transcript levels. (E) Immunoblotting of FKBP51 and FKBP52 in control and knockdown cell lines.

There was no significant effect on the AR-regulated *TMPRSS2* gene (Fig. 3C). Transcript levels for the *KLK2*, *PSA*, and *S100P* genes were reduced in CWR22Rv1 cells that contained the FKBP51 shRNA (Fig. 3D), an AI derivative of CWR22 that overexpresses FKBP51 (17). These data provide clear evidence that FKBP51 contributes to the regulation of AR activity in prostate cancer cells. This is despite the fact that FKBP51 is not required for AR function in the development of the male mouse reproductive system, which instead relies on the closely related cochaperone FKBP52 (4, 37).

FKBP51 expression enhances AR responsiveness to diverse ligands. Overexpression of AR increases transcription and growth in response to the antiandrogen Casodex (Cas) (3). Reasoning that FKBP51 regulates some aspect of the AR ligand-binding cycle, we analyzed whether its overexpression alters AR-dependent transcription in response to several AR-binding ligands. FKBP51 stimulated AR transcriptional activity in the presence of Cas, cyproterone acetate, and hydroxyflutamide, while little enhancement was observed in the presence of flutamide (Fig. 4A). FKBP51 also stimulated AR activity in the presence of the adrenal androgen androstenedione and high concentrations of estrogen, but not of Dex (Fig. 4A). These results prompted us to explore whether FKBP51 affects the efficacy of the antiandrogen Cas, which is used as a competitive inhibitor of adrenal androgens in patients undergoing androgen ablation therapy. We tested this by measuring AR transcription activity in the presence of synthetic androgen (R1881) and a range of Cas concentrations.

F4

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

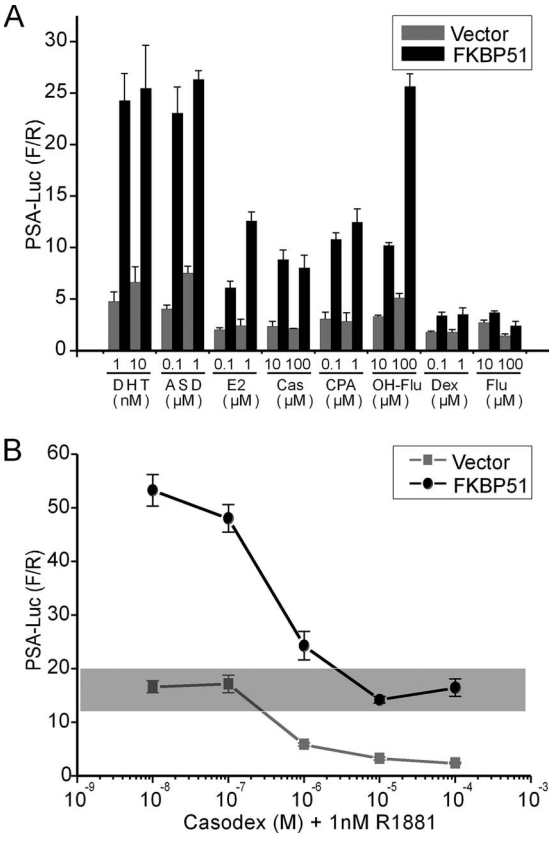


FIG. 4. AR activation in response to diverse ligands is enhanced by FKBP51. (A) AR activity in response to the indicated ligands is modulated by FKBP51. Activity was measured in PC-3 cells by using the reporter gene PSA-Luc. (B) AR activity in the presence of R1881 (1 nM) as a function of the Cas concentration was measured by using the reporter gene PSA-Luc. CPA, cyproterone acetate; Flu, flutamide; OH-Flu, hydroxyflutamide; ASD, androstenedione; DHT, dihydrotestosterone; E2, estrogen.

While FKBP51 did not change the apparent K_i for Cas, it did render AR resistant to maximal inhibition by Cas (Fig. 4B). In fact, the level of AR activity measured in the presence of FKBP51 overexpression and a saturating concentration of Cas (10 to 100 μM) was comparable to the level of AR activity measured simply in the presence of R1881 (gray shading). These data show that FKBP51 overexpression promotes AR activity in response to a variety of ligands, and it has the potential to influence the efficacy of antiandrogens used in prostate cancer treatment.

FKBP51 expression alters the chaperone composition of AR complexes. To gain insight into how FKBP51 regulates AR, we analyzed the chaperone composition of AR complexes from PC-3 cells cotransfected with FKBP51. Flag-AR complexes were isolated by IP under nondenaturing conditions, and molybdate was included to preserve chaperone interactions. Chaperones that coprecipitated with Flag-AR included Hsp90, Hsp70, FKBP51, and p23 (Fig. 5A). FKBP51 induced a striking increase in the level of chaperones in AR complexes, particularly p23 and Hsp90 (Fig. 5A). We viewed this effect as potentially significant because the cochaperone p23 binds directly to the ATP form of Hsp90, and this is known to stabilize Hsp90-client protein interactions (1, 14, 15). In this context, p23 is known to mark steroid receptors in the apo state that are poised to bind hormone (15). These data imply that in prostate cancer cells, FKBP51 levels may be limiting for the formation of p23-Hsp90-client protein complexes.

FKBP51 promotes p23 binding to Hsp90. Our data suggested that FKBP51 might control AR activity by regulating chaperone complex assembly. Such a pathway could help define the number of AR molecules that attain the conformation required for the LBD to bind androgen. To more clearly define the contribution of FKBP51 in this pathway, we used recombinant proteins to test whether FKBP51 regulates chaperone complex assembly in a purified system. Hsp90, p23, and FKBP51 (1 μM each) were incubated alone and in various combinations. The samples were then subjected to gel filtration chromatography, and the eluted fractions were examined by immunoblotting. In this assay, protein assembly into the chaperone complex results in coelution with Hsp90. We found that p23 interacted weakly with Hsp90 (Fig. 5B, lanes 3 and 4, upper panels), but the interaction was enhanced markedly by the addition of FKBP51 and resulted in the formation an FKBP51-p23-Hsp90 ternary complex (Fig. 5B, lanes 3 and 4, lower panels).

Using the gel filtration assay, we examined the nucleotide requirements and effects of inhibitors on FKBP51-p23-Hsp90 ternary complex formation *in vitro*. Nucleotides and inhibitors were added to the assembly reaction mixtures prior to chromatography. The amount of p23 coeluting with Hsp90 was quantified and plotted as histograms. Assembly was supported by ATP or AMP-PNP, indicating a requirement for ATP binding to Hsp90 but not hydrolysis. Moreover, ternary complex assembly could be inhibited by geldanamycin (GA) and FK506, drugs that bind to Hsp90 and FKBP51, respectively (Fig. 5C). We corroborated the results of our chaperone assembly assays (with recombinant proteins) by isolating chaperone complexes from cultured cells. Following a brief (1-h) treatment of cells with GA and FK506, FKBP51 complexes were isolated by IP and the chaperone content was analyzed by immunoblotting. Treatment with GA essentially abolished p23 recruitment into the FKBP51-Hsp90 complex, though it did not appear to affect Hsp90 binding to FKBP51 (Fig. 5D, lane 3). FK506 reduced the levels of both Hsp90 and p23 associated with FKBP51 (Fig. 5D, lane 4). Our data suggest that Hsp90 mediates the interaction between FKBP51 and p23. We tested this by using recombinant proteins and gel filtration chromatography. When Hsp90 is omitted from the binding reaction mixture, FKBP51 and p23 do not form a complex (Fig. 5E). These data suggest that FKBP51 modulates p23 binding to Hsp90 through its contact with Hsp90 and not by direct contact with p23.

FKBP51 increases the number of androgen-bound AR molecules in the cell. Since Hsp90 binds the LBD of steroid hormone receptors (24) and FKBP51 promotes Hsp90 ternary complex assembly, we anticipated that FKBP51 would regulate the LBD of AR. We tested this by deleting the LBD of AR and analyzing the chaperone content of AR isolated by IP. Neither FKBP51 nor p23 was detected in AR complexes when the LBD was deleted from AR (Fig. 6). Our observations, together with the established role of Hsp90 in the steroid hormone-binding cycle, led us to hypothesize that FKBP51 promotes a step in the AR ligand-binding cycle. To formally address whether

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

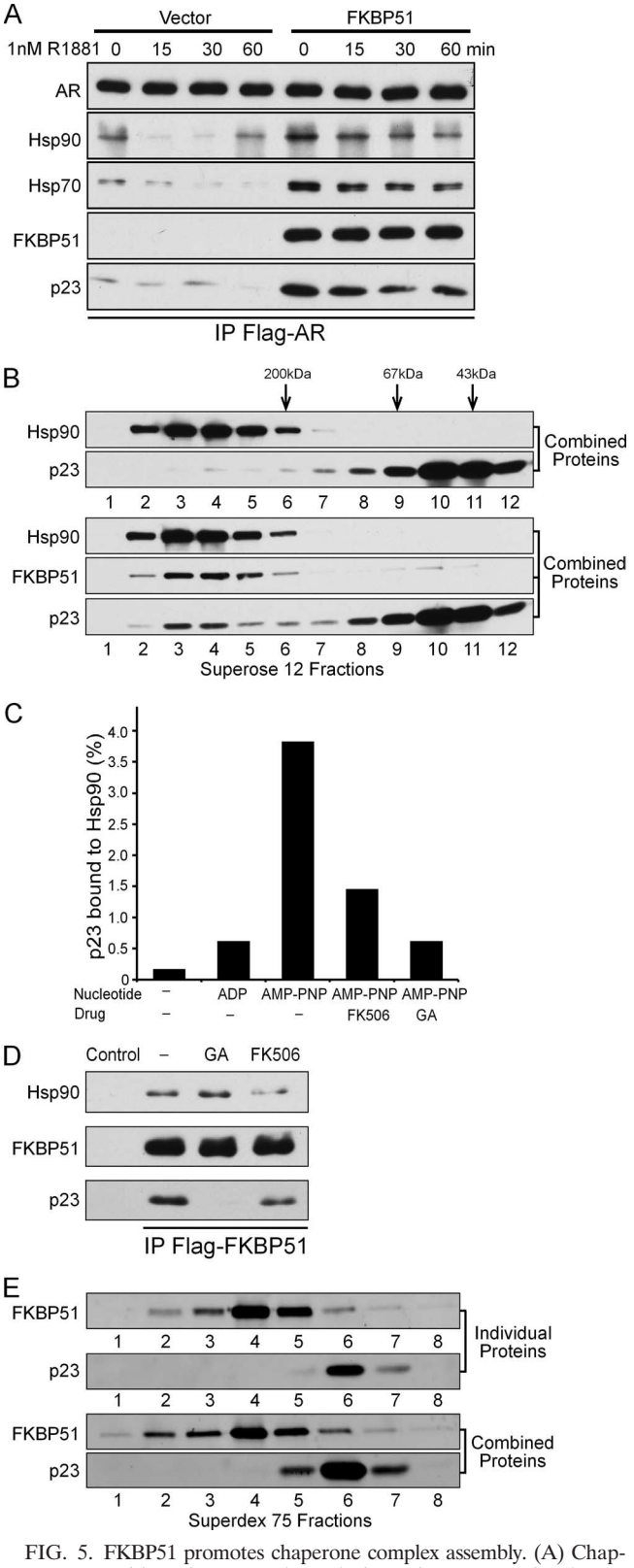


FIG. 5. FKBP51 promotes chaperone complex assembly. (A) Chaperone composition of AR complexes during a time course of androgen treatment. PC-3 cells were transfected with plasmids encoding AR and FKBP51 and treated with R1881 for the times indicated. AR complexes were isolated by IP and immunoblotted for chaperones. (B) *In vitro* assembly of chaperone complexes. Recombinant chaperone proteins (individually and in various combinations) were separated by gel filtration on a Superose 12 column, and their elution positions were determined by immunoblotting to evaluate complex formation. The elution profiles of individual proteins are not shown. (C) Effects of nucleotides and inhibitors on p23 assembly into the Hsp90 complex. Nucleotides (2 mM each) and chaperone inhibitors (10 μM GA, 10 μM FK506) were included in the chaperone complex assembly reaction mixtures as indicated. The reaction products were then applied to a Superose 12 column, and the amount of p23 in the FKBP51-Hsp90 complex was determined relative to the total amount of p23 in the reaction mixture by immunoblotting (see Materials and Methods). (D) FKBP51-p23-Hsp90 complex formation in cells is sensitive to inhibition by GA and FK506. LNCaP cells stably expressing Flag-FKBP51 were treated with inhibitors (1 μM GA, 10 μM FK506) for 1 h. Complexes were then isolated by IP and analyzed by immunoblotting. (E) Evaluation of cochaperone interactions in the absence of Hsp90. FKBP51 and p23 (as individual and combined proteins) were separated by gel filtration on a Superdex 75 column. FKBP51 and p23 fail to assemble into a complex unless the reaction mixture contains Hsp90 (compare with panel B).

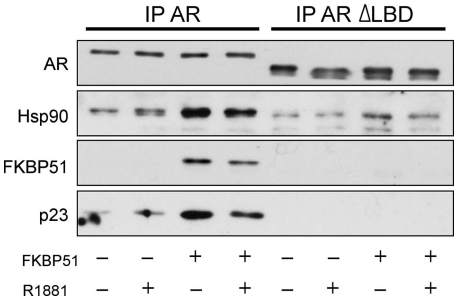


FIG. 6. The LBD is required for AR association with the FKBP51-p23-Hsp90 superchaperone complex. PC-3 cells were transfected with full-length AR and AR lacking the LBD (ARΔLBD) and FKBP51 (or empty vector). After androgen treatment, AR complexes were isolated by IP and analyzed for chaperone content by immunoblotting.

FKBP51 affects androgen binding to AR, we transfected AR into AR-negative PC-3 cells without and with FKBP51 and performed whole-cell hormone-binding assays. Remarkably, FKBP51 expression increased the number of androgen-binding sites by 38% without changing AR levels (Fig. 7A). Moreover, FKBP51 increased androgen binding in the whole-cell assay without increasing the affinity of AR for androgen (Fig. 7A).

To establish that the increase in androgen binding in the whole-cell assay was due to androgen binding directly to AR (as opposed to a cellular factor whose expression was induced by AR), we used a quantitative coimmunoprecipitation assay developed in our laboratory (35) that discriminates between androgen-free and androgen-bound forms of AR. In the presence of SV40 small t antigen, protein phosphatase 2A (PP2A) undergoes highly selective loading onto androgen-bound AR (35). Using the PP2A-AR interaction assay, we found that FKBP51 expression approximately doubled the ratio of PP2A to AR in an immunoprecipitate from cells treated with androgen. Treating cells with FK506 blocked the stimulatory effect of FKBP51 in this assay, but it did not reduce the basal level of androgen binding to AR (Fig. 7B). This result suggests that, even in the absence of FKBP51 activity, a pool of AR molecules acquire competence for androgen binding. While neither

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

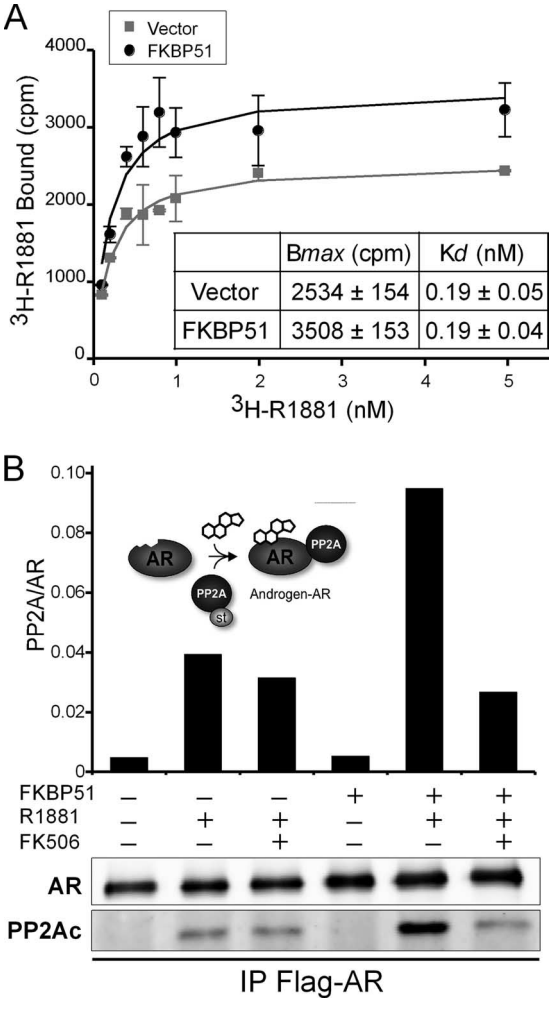


FIG. 7. FKBP51 increases the number of AR molecules that bind androgen. (A) FKBP51 regulates androgen binding to AR. Whole-cell [^{3}H]R1881-binding assays were performed with PC-3 cells transfected with AR with or without FKBP51. (B) PP2A interaction assay for measuring the relative number of androgen-bound AR molecules. PP2A undergoes selective transfer from small t antigen onto AR because the PP2A A subunit recognizes features of the LBD that are unique to the agonist-bound form (35). FKBP51 increases the number of AR molecules that bind androgen, resulting in an increase in the PP2A/AR ratio. FK506 inhibits FKBP51-stimulated formation of PP2A-AR complexes and FKBP51 enhancement of AR-dependent transcription (see Fig. 2F).

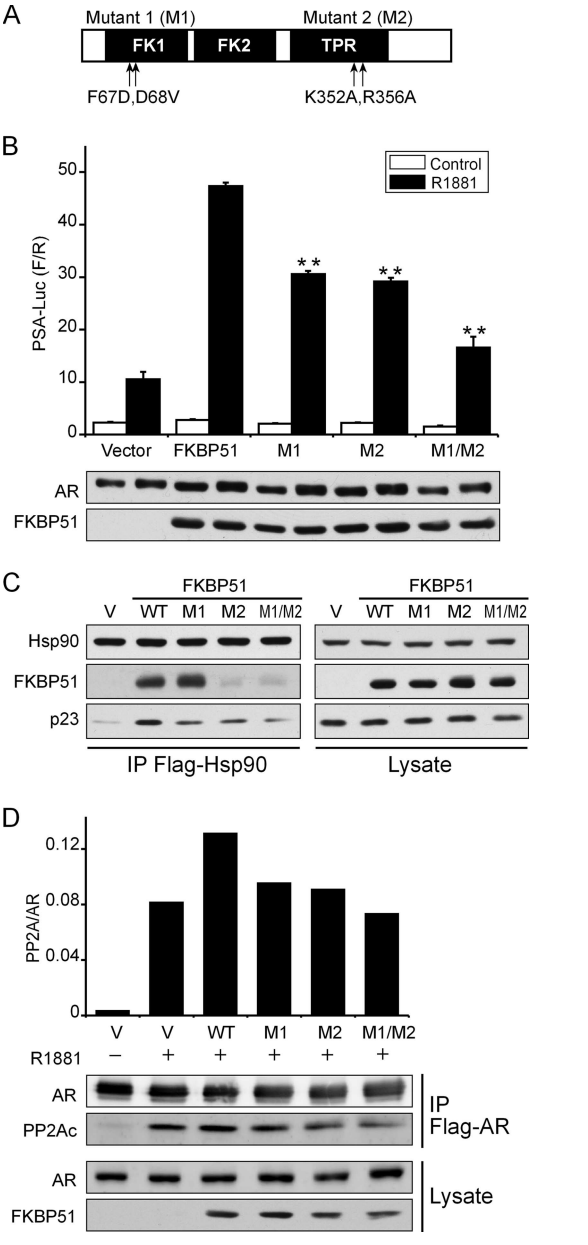
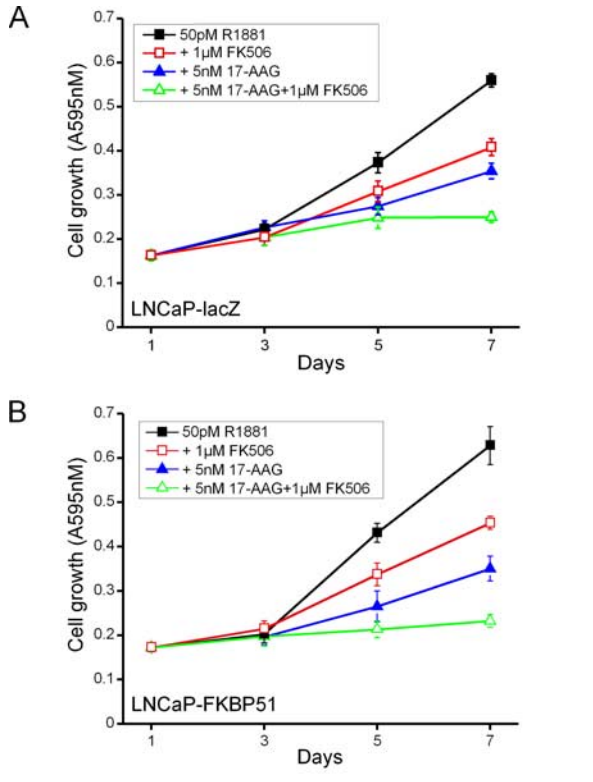


FIG. 8. Point mutations that decrease FKBP51 activity reduce its ability to enhance AR-dependent transcription and superchaperone complex assembly. (A) Diagram of FKBP51 showing the three major structural domains (black shading) and the positions of the point mutations that disrupt prolyl isomerase activity (M1; F67D, D68V) and binding to Hsp90 (M2; K352A, R356A). The M1/M2 form of FKBP51 contains both sets of point mutations. (B) PSA reporter assay with wild-type and mutant forms of FKBP51. Asterisks denote P values (**, $P < 0.01$) comparing the activities of mutant forms of FKBP51 to that of wild-type FKBP51. The mutant forms of FKBP51 show the same reductions in activity relative to wild-type FKBP51 when the data are plotted as fold activation induced by R1881 (not shown). (C) Effects of FKBP51 point mutations on enhancement of superchaperone complex assembly. PC-3 cells were cotransfected with Flag-Hsp90 and untagged forms of FKBP51. Hsp90 complexes were isolated by IP and immunoblotted for chaperones (transfected FKBP51, endogenous p23). (D) Analysis of mutant forms of FKBP51 by using the PP2A-AR to assess the effect on androgen binding to AR. The amount of PP2A that coimmunoprecipitates with AR was plotted as a PP2A/AR ratio. The FKBP51 enhancement of androgen binding to AR obtained with (wild-type protein) was reduced in the M1, M2, and M1/M2 mutant forms of FKBP51. V, vector.

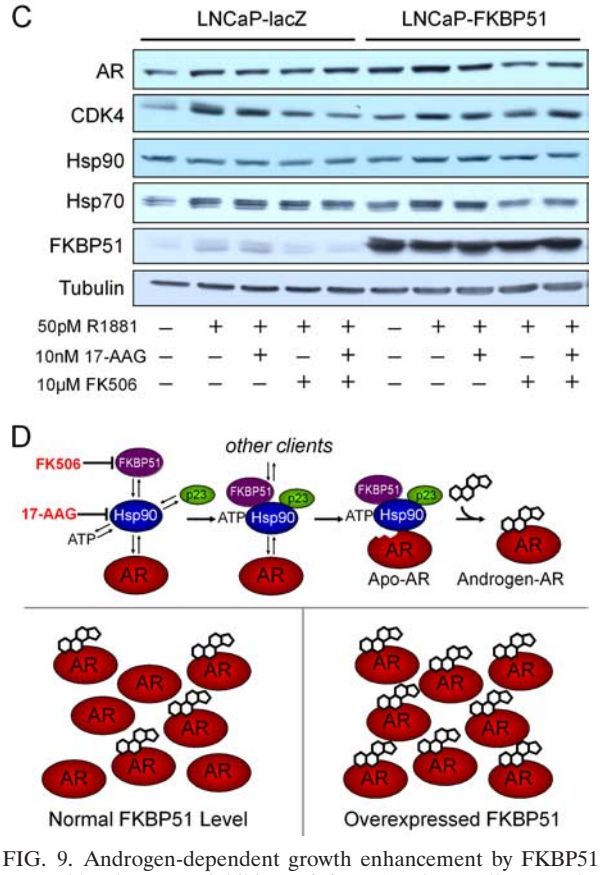
FKBP51 nor FKBP52 is required for the androgen-binding cycle of AR, the expression of these cochaperones is critical for physiological levels of AR signaling during development and in prostate cancer. In summary, our data show that FKBP51 promotes the assembly of a superchaperone-AR complex marked by p23, and this leads to a substantial increase in the number of androgen-bound AR molecules in the cell. FKBP51 overexpression therefore provides a pathway for increasing AR signaling, which is known to promote AI-type growth in human prostate cancer models (3).

FKBP51 activity toward AR is dependent on prolyl isomerase activity and Hsp90 binding. We used mutant forms of FKBP51 to determine whether prolyl isomerase activity and

F8



F9



4 COLOR

FIG. 9. Androgen-dependent growth enhancement by FKBP51 is prevented by chaperone inhibitors. (A) LNCaP-lacZ cells were plated and grown in the absence or presence of a low androgen concentration (50 pM R1881). Inhibitors of FKBP51 (FK506, 1 μM) and Hsp90 (17-AAG, 5 nM) were added alone and in combination. (B) LNCaP-FKBP51 cells were prepared and treated by the aforementioned methods. (C) Levels of AR, CDK4, Hsp90, Hsp70, FKBP51, and tubulin in LNCaP cells treated with the indicated combinations of drugs and ligand. Blockage of androgen-induced growth by dual treatment with FK506 and 17-AAG (4 days) does not cause significant changes in the Hsp90 client protein CDK4 or Hsp70. (D) Summary of protein interactions and model of FKBP51 function in AI prostate cancer. Hsp90 and other chaperones undergo dynamic interactions with nuclear receptors. Assembly of the FKBP51-Hsp90-p23 superchaperone complex in association with the LBD of AR is proposed to be a critical step for acquiring hormone-binding competence. Promoting superchaperone complex assembly, in turn, increases the number of AR molecules that bind androgen. This provides a mechanism for AR-dependent signaling and growth under low-androgen conditions.

Hsp90 binding is important for FKBP51 stimulation of AR activity on the *PSA* promoter. The M1 mutant form characterized by Smith and coworkers (2) has two amino acid changes in the FK1 domain that reduce prolyl isomerase activity. Mutations in the TPR domain that disrupt binding to Hsp90, described originally by Chinkers and associates in PP5 (29), were also constructed (M2). FKBP51 enhancement of AR activity measured on the *PSA* promoter was impaired by the M1 and M2 mutations, which, in combination (M1/M2), gave a larger reduction in FKBP51 activity toward AR (Fig. 8B). The prolyl isomerase mutation (M1) reduced the ability of FKBP51 to promote p23 assembly into the Hsp90 chaperone complex without affecting FKBP51 binding to Hsp90 (Fig. 8B). Finally, the M1, M2, and M1/M2 mutant forms have reduced activity in the PP2A-AR interaction assay, which is a readout of the number of AR molecules that are bound with androgen (Fig. 8C). Our data indicate that physical contact with Hsp90 (via the TPR domain) and the prolyl isomerase activity (in the FK1 domain) are important for chaperone complex assembly and enhancement of AR activity.

17-AAG and FK506 inhibit androgen-stimulated cell growth. Inhibitors of Hsp90 are promising therapeutics for cancer because Hsp90 regulates client proteins critical for signal transduction and cell growth (20). The Hsp90 inhibitor 17-AAG is a GA derivative with reduced hepatotoxicity in stage II clinical trials for cancers, including prostate cancer (34). 17-AAG and the immunophilin ligand FK506 each can inhibit prostate cancer cell growth in culture (21, 30). Given our finding that *in vitro* assembly of the Hsp90-p23-FKBP51 superchaperone complex was prevented by inhibitors of Hsp90 and FKBP51 (Fig. 5C), we tested whether chaperone inhibitors were effective in blocking prostate cancer cell growth in response to androgen. 17-AAG reduced the androgen-stimulated growth of FKBP51-overexpressing and control cells to similar extents, showing that Hsp90 inhibition is sufficient to overcome the growth benefit provided by FKBP51 (Fig. 9A and B). Treating cells with a combination of 17-AAG (5 nM) and FK506 (1 μM) resulted in an additive effect on growth inhibition in both the control and FKBP51-overexpressing cells (Fig. 9A and B). Other groups have shown that high concentrations of 17-AAG inhibit AR by affecting protein stability (30); however, AR levels did not change significantly when cells were maintained in low concentrations of 17-AAG (10 nM) and FK506 (10 μM) for 4 days. CDK4, an established client of Hsp90, also showed

minimal change in response to 17-AAG and FK506 treatment (Fig. 9C). Our data suggest that a drug combination that targets the Hsp90 superchaperone complex can effectively inhibit androgen-stimulated growth of prostate cancer cells without causing major changes in CDK4 and Hsp70 levels.

DISCUSSION

Though androgen ablation remains the cornerstone of prostate cancer treatment, prostate cancer patients develop resistance to this therapy. Prostate cancer progresses from localized AD tumors to metastatic AI disease with an attendant poor prognosis. Defining the mechanisms that drive AI disease is therefore one of the major goals of prostate cancer research. We took a highly focused approach that involved testing whether the expression of protein chaperones with known links to steroid hormone binding are altered in AI prostate cancer. Utilizing the LAPC-4 xenograft system developed by Sawyers and colleagues (3), we found that the cochaperone FKBP51 is overexpressed in AI xenografts propagated in castrated mice. FKBP51 is a peptidyl-prolyl isomerase that binds the immunosuppressants FK506 and rapamycin. FKBP51 clearly antagonizes GR and PR function by decreasing binding affinity for hormone, but its specific mode of action on Hsp90 and steroid hormone receptors has been enigmatic. In mouse prostate and in human prostate cancer cells, FKBP51 expression is tightly regulated by androgen. In the CWR22 xenograft, FKBP51 expression is strongly reduced upon host castration but FKBP51 levels in the recurrent tumor recover to a level that exceeds those in the primary tumor (16). We found the same trend in LAPC-4 xenografts, with significantly higher FKBP51 expression levels at both the protein and mRNA levels in the AI LAPC-4 tumor grown in castrated mice compared with AD tumors grown in intact mice. These results obtained with CWR22 and LAPC-4 xenografts could reflect FKBP51 expression changes during human prostate cancer progression. In a sampling of 52 local and 9 metastatic prostate cancers, Febbo and coworkers (7) reported that FKBP51 displays a metastatic-to-local tumor expression ratio of 2.9.

Because FKBP51 transcription is regulated by AR, a key question that arose is whether the elevation of FKBP51 transcript and protein levels in the AI state reflects enhanced AR activity or whether the FKBP51 protein promotes AR activity. We found that FKBP51 promotes AR-dependent transcription using both reporter- and endogenous-gene-based assays, consistent with published data (7). Moreover, FKBP51 enhanced LNCaP cell growth in response to a low androgen concentration (50 pM). The growth benefit of FKBP51 in LNCaP cells was inhibited by 17-AAG, which links FKBP51 to Hsp90 function. We obtained direct evidence that FKBP51 induces assembly of the Hsp90 superchaperone complex and that this regulates the LBD of AR (Fig. 9D). This involves FKBP51 contact with Hsp90 and recruitment of p23, a cochaperone known to stabilize Hsp90 binding to client proteins (14). Crystallographic analysis revealed that p23 recognizes the ATP-bound, closed conformation of Hsp90; a major contact surface for p23 is formed by "lid" closure of Hsp90 (1). Thus, FKBP51 may promote ATP binding or lid closure or stabilize another mobile element in the Hsp90 structure such as the catalytic loop that is contacted by p23 (1).

Hsp90 inhibitors are promising as therapeutics because Hsp90 regulates client proteins, including AR, that are critical for signal transduction and cell growth in cancer (20). High-dose 17-AAG, however, causes skeletal complications and increases Hsp70 levels, the latter of which may promote tumor resistance to therapy (23, 25). The use of Hsp90 inhibitors in combination with drugs that target cochaperones that function in the same multisubunit complex is a potential strategy for inhibiting clients such as AR that are highly dependent on the superchaperone complex.

By promoting Hsp90 superchaperone complex assembly, FKBP51 has a quantitative effect on androgen signaling by increasing the number of AR molecules that are subsequently loaded with hormone (Fig. 9D). Because androgen-bound AR regulates FKBP51 expression, this creates a feed-forward mechanism that could amplify AR signaling under the low-hormone conditions that occur during androgen ablation.

ACKNOWLEDGMENTS

We thank David Smith for providing FKBP51 reagents, Charles Sawyers for LAPC-4 cells, Len Neckers for Hsp90 constructs, Hongwu Xin and Adam Spencer for technical assistance, and members of the Paschal lab for helpful discussions.

REFERENCES

AQ: D

1. **Ali, M. M., S. M. Roe, C. K. Vaughan, P. Meyer, B. Panaretou, P. W. Piper, C. Prodromou, and L. H. Pearl.** 2006. Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature **440:**1013–1017.
2. **Barent, R. L., S. C. Nair, D. C. Carr, Y. Ruan, R. A. Rimerman, J. Fulton, Y. Zhang, and D. F. Smith.** 1998. Analysis of FKBP51/FKBP52 chimeras and mutants for Hsp90 binding and association with progesterone receptor complexes. Mol. Endocrinol. **12:**342–354.
3. **Chen, C. D., D. S. Welsbie, C. Tran, S. H. Baek, R. Chen, R. Vessella, M. G. Rosenfeld, and C. L. Sawyers.** 2004. Molecular determinants of resistance to antiandrogen therapy. Nat. Med. **10:**33–39.
4. **Cheung-Flynn, J., V. Prapapanich, M. B. Cox, D. L. Riggs, C. Suarez-Quian, and D. F. Smith.** 2005. Physiological role for the cochaperone FKBP52 in androgen receptor signaling. Mol. Endocrinol. **19:**1654–1666.
5. **Dehm, S. M., and D. J. Tindall.** 2007. Androgen receptor structural and functional elements: role and regulation in prostate cancer. Mol. Endocrinol. **21:**2855–2863.
6. **Denny, W. B., D. L. Valentine, P. D. Reynolds, D. F. Smith, and J. G. Scammell.** 2000. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology **141:**4107–4113.
7. **Febbo, P. G., M. Lowenberg, A. R. Thorner, M. Brown, M. Loda, and T. R. Golub.** 2005. Androgen mediated regulation and functional implications of fkbp51 expression in prostate cancer. J. Urol. **173:**1772–1777.
8. **Freeman, B. C., and K. R. Yamamoto.** 2002. Disassembly of transcriptional regulatory complexes by molecular chaperones. Science **296:**2232–2235.
9. **Hubler, T. R., W. B. Denny, D. L. Valentine, J. Cheung-Flynn, D. F. Smith, and J. G. Scammell.** 2003. The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness. Endocrinology **144:**2380–2387.
10. **Johnson, J. L., T. G. Beito, C. J. Krco, and D. O. Toft.** 1994. Characterization of a novel 23-kilodalton protein of unactive progesterone receptor complexes. Mol. Cell. Biol. **14:**1956–1963.
11. **Kesler, C. T., D. Gioeli, M. R. Conaway, M. J. Weber, and B. M. Paschal.** 2007. Subcellular localization modulates activation function 1 domain phosphorylation in the androgen receptor. Mol. Endocrinol. **21:**2071–2084.
12. **Klein, K. A., R. E. Reiter, J. Redula, H. Moradi, X. L. Zhu, A. R. Brothman, D. J. Lamb, M. Marcelli, A. Belldegrun, O. N. Witte, and C. L. Sawyers.** 1997. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat. Med. **3:**402–408.
13. **Magee, J. A., L. W. Chang, G. D. Stormo, and J. Milbrandt.** 2006. Direct, androgen receptor-mediated regulation of the FKBP5 gene via a distal enhancer element. Endocrinology **147:**590–598.
14. **McLaughlin, S. H., F. Sobott, Z. P. Yao, W. Zhang, P. R. Nielsen, J. G. Grossmann, E. D. Laue, C. V. Robinson, and S. E. Jackson.** 2006. The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. J. Mol. Biol. **356:**746–758.
15. **Morishima, Y., K. C. Kanelakis, P. J. Murphy, E. R. Lowe, G. J. Jenkins, Y. Osawa, R. K. Sunahara, and W. B. Pratt.** 2003. The hsp90 cochaperone p23

zmb00510/zmb8488d10z | xppws | S=1 | 1/7/10 | 4/C Fig: 1,9 | Art: 1891-08

is the limiting component of the multiprotein hsp90/hsp70-based chaperone system in vivo where it acts to stabilize the client protein: hsp90 complex. J. Biol. Chem. **278:**48754–48763.

16. **Mousses, S., L. Bubendorf, U. Wagner, G. Hostetter, J. Kononen, R. Cornelison, N. Goldberger, A. G. Elkahloun, N. Willi, P. Koivisto, W. Ferhle, M. Raffeld, G. Sauter, and O. P. Kallioniemi.** 2002. Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. Cancer Res. **62:**1256–1260.
17. **Nagabhushan, M., C. M. Miller, T. P. Pretlow, J. M. Giaconia, N. L. Edgehouse, S. Schwartz, H. J. Kung, R. W. de Vere White, P. H. Gumerlock, M. I. Resnick, S. B. Amini, and T. G. Pretlow.** 1996. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res. **56:**3042–3046.
18. **Nair, S. C., R. A. Rimerman, E. J. Toran, S. Chen, V. Prapapanich, R. N. Butts, and D. F. Smith.** 1997. Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. Mol. Cell. Biol. **17:**594–603.
19. **Pearl, L. H., and C. Prodromou.** 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu. Rev. Biochem. **75:**271–294.
20. **Pearl, L. H., C. Prodromou, and P. Workman.** 2008. The Hsp90 molecular chaperone: an open and shut case for treatment. Biochem. J. **410:**439–453.
21. **Periyasamy, S., M. Warrier, M. P. Tillekeratne, W. Shou, and E. R. Sanchez.** 2007. The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and -independent mechanisms. Endocrinology **148:**4716–4726.
22. **Plymate, S. R., M. K. Tennant, S. H. Culp, L. Woodke, M. Marcelli, I. Colman, P. S. Nelson, J. M. Carroll, C. T. J. Roberts, and J. L. Ware.** 2004. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF-I on proliferation and AR activity between localized and metastatic tumors. Prostate **61:**276–290.
23. **Powers, M. V., P. A. Clarke, and P. Workman.** 2008. Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis. Cancer Cell **14:**250–262.
24. **Pratt, W. B., and D. Toft.** 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. **18:**306–360.
25. **Price, J. T., J. M. W. Quinn, N. A. Sims, J. Vieusseux, K. Waldeck, S. E. Docherty, D. Myers, A. Nakamura, M. C. Waltham, M. T. Gillespie, and E. W. Thompson.** 2005. The heat shock protein 90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, enhances osteoclast formation and potentiates bone metastasis of a human breast cancer cell line. Cancer Res. **65:**4929–4938.
26. **Ratajczak, T., B. K. Ward, and R. F. Minchin.** 2003. Immunophilin chaperones in steroid receptor signalling. Curr. Top. Med. Chem. **3:**1348–1357.
27. **Riggs, D. L., M. B. Cox, H. L. Tardif, M. Hessling, J. Buchner, and D. F. Smith.** 2007. Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. Mol. Cell. Biol. **27:**8658–8669.
28. **Riggs, D. L., P. J. Roberts, S. C. Chirillo, J. Cheung-Flynn, V. Prapapanich, T. Ratajczak, R. Gaber, D. Picard, and D. F. Smith.** 2003. The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. EMBO J. **22:**1158–1167.
29. **Russell, L. C., S. R. Whitt, M. S. Chen, and M. Chinkers.** 1999. Identification of conserved residues required for the binding of a tetratricopeptide repeat domain to heat shock protein 90. J. Biol. Chem. **274:**20060–20063.
30. **Saporita, A. J., J. Ai, and Z. Wang.** 2007. The Hsp90 inhibitor, 17-AAG, prevents the ligand-independent nuclear localization of androgen receptor in refractory prostate cancer cells. Prostate **67:**509–520.
31. **Shank, L. C., and B. M. Paschal.** 2005. Nuclear transport of steroid hormone receptors. Crit. Rev. Eukaryot. Gene Expr. **15:**49–73.
32. **Smith, D. F., W. P. Sullivan, T. N. Marion, K. Zaitsu, B. Madden, D. J. McCormick, and D. O. Toft.** 1993. Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. Mol. Cell. Biol. **13:**869–876.
33. **Sullivan, W. P., B. A. Owen, and D. O. Toft.** 2002. The influence of ATP and p23 on the conformation of hsp90. J. Biol. Chem. **277:**45942–45948.
34. **Workman, P.** 2004. Altered states: selectively drugging the Hsp90 cancer chaperone. Trends Mol. Med. **10:**47–51.
35. **Yang, C.-S., H.-W. Xin, J. B. Kelley, A. Spencer, D. L. Brautigan, and B. M. Paschal.** 2007. Ligand binding to the androgen receptor induces conformational changes that regulate phosphatase interactions. Mol. Cell. Biol. **27:**3390–3404.
36. **Yeh, W. C., T. K. Li, B. E. Bierer, and S. L. McKnight.** 1995. Identification and characterization of an immunophilin expressed during the clonal expansion phase of adipocyte differentiation. Proc. Natl. Acad. Sci. U. S. A. **92:**11081–11085.
37. **Yong, W., Z. Yang, S. Periyasamy, H. Chen, S. Yucel, W. Li, L. Y. Lin, I. M. Wolf, M. J. Cohn, L. S. Baskin, E. R. Sanchez, and W. Shou.** 2007. Essential role for cochaperone Fkbp52 but not Fkbp51 in androgen receptor-mediated signaling and physiology. J. Biol. Chem. **282:**5026–5036.